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Original Article

Cloning of the Gene Encoding *M2e* of Influenza Virus in *B. subtilis*

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Abstract

Background and Aims: The ectodomain of matrix protein of influenza virus is a weak immunogen that is highly conserved among all subtypes of influenza A virus. Tandem repeats of these genes along with linker were used to enhance immunogenicity of M2e protein and so it can be served as a universal vaccine in both humans and livestock.

Materials and Methods: In this study, the sequences of extra-domain of matrix protein of influenza A registered in NCBI was converted into codons compatible for *Bacillus subtilis* using JAVA codon adaptation tool software.

Results: A cassette consist of four repeats of this codon optimized sequence, spaced by appropriate linkers and flanked by *BamHI* and *HindIII* restriction sites was designed and thoroughly used for the synthesis. The cassette then was cloned into pMR12 shuttle expression vector.

Conclusion: Two kinds of prokaryotic host, *E. coli* BL21and *Bacillus subtilis* WB600 were transformed by pMR12+4M2e. The fidelity of the construct in both transformants was confirmed by enzymatic analysis and PCR.

Keywords: Influenza A; Matrix protein 2; Cloning; *Bacillus subtilis*; pMR12

Introduction

Orthomyxoviridae contains four genera: Influenza virus A, Influenza virus B, *Influenza virus C*, and *Thogotovirus*. Envelope protein of Orthomyxoviridae has hemagglutinating properties.Their genomes are segmented negative-sense RNA that lead to antigenic variation(1-4).Both influenza A and B viruses possess eight pieces of segmented single stranded RNA that have a total length of about 13600 and 14600 nucleotides, respectively (5). Influenza A viruses are classified, based on the viral surface proteins hemagglutinin (HA) and neuraminidase (NA). Up to now, Sixteen HA subtypes and nine NA subtypes of influenza A viruses have been identified. The source of all influenza Aviruses of animal, human and aquatic mammals is aquatic birds and all types of influenza viruses have been isolated from birds (6-9). Epidemics and pandemics process of influenza viruses are approvement of antigenic variation of the virus (1). Genetic variations such as genetic reassortment, genetic recombination, substitution mutations, insertion mutations, deletion mutations and point mutations have been reported among influenza viruses. Crossing the species barriers is facilitated by antigenic variation in influenza virus. Influenza A virus infects birds, humans, livestock, rodents and some of the aquatic animals where as influenza B and C viruses mainly affect

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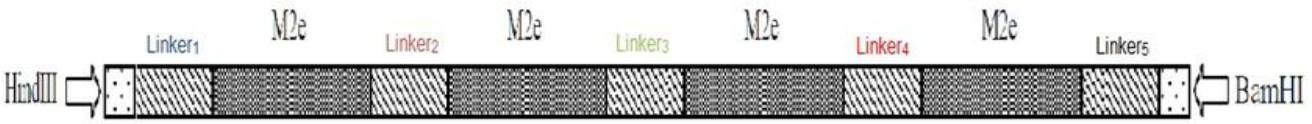

Fig. 1. Schematic view of a designed construct.a tandem repeat of 4 copies of M2e (4*M2e*).

humans (10). Current influenza vaccines are largely based on viral hemagglutinin (HA) and neuraminidase (NA) proteins. Genetic instability of HA and NA glycoproteins is one of the main challenges in the production of current vaccines, therefore new pathogenic strains emerge each year and the flu vaccine needs to be reformulated to keep up with the changes in the circulating strains. Accordingly each year a new vaccine is being marketed with a new formula that many of them may not have any effect on preventing influenza illness. An effective vaccine must contain influenza antigens that are highly conserved among the different types of influenza viruses. The vaccines which are produced based on such antigens do not require to predict circulating strains during a season and it can prevent the hastily manufactured vaccines (11). M2 is a surface layer of matrix proteins of the influenza A virus which functions as an ion channel. M2 is a promising candidate for development of a universal influenza vaccine which may provide cross-protection against different strains of influenza A viruses.The M2 protein consists of 96 amino acid residues which the 23-amino acid extracellular domain of M2, known as M2e. This protein is relatively invariant from strain to strain of influenza A viruses and does not undergo drift and shift mutations therefore is considered as a good choice for development of a vaccine to provide broad protection (5, 12). This antigen alone is not very immunogenic due to its small size, thus, different adjuvants or other antigens can be utilized to increase its immunogenicity (9, 13). The purpose of this study is cloning of a tandem repeat of 4 copies of *M2e* (4×M2e) along with suitable linkers by fusion method in *B. subtilis*.

Methods

Design and synthesis of cassette

All the sequences of *M2e* protein were obtained from NCBI and were compared with each other, then, four M2e protein sequences using five different linkers were attached to each other. The linkers were designed by Linker DB software and http://Chutey.med.yale.edu/Linker/linker.html website. The amino acid sequence of assembled polypeptide was converted into codons which are compatible for *Bacillus subtilis* by using JAVA codon adaptation tool and http://www.genscript.com/cgi-bin/tools/rare_codon_analysis website. Cleavage sites for enzymes *HindIII* and *BamHI* were inserted at the beginning and the end of the construct. Designed construct was synthesized by Macrogen Company (Korea) and delivered on the pUC57 vector.

Gene cloning in *E. coli*

The pUC57 vector containing the construct and pMR12 shuttle expression vector (14) were cleaved with enzymes *BamHI* (Enzynomics, Korea) and Fast Digest *HindIII* (Fermentas, Ukraine). Digested construct and vector were isolated from gel by gel extraction kit (Fermentas, Ukraine) and then, ligation reaction were performed between 800ng of construct and 200ng of pMR12 vector according to the standard methods by enzyme T4 DNA Ligase (Enzynomics, Korea) .The ligation reaction was transferred to *E. coli BL21*by cold calcium chloride treatment and 100µl of transferred mixture was cultured on LB agar medium containing ampicillin (100 µl/ml) (Sigma USA) (15).

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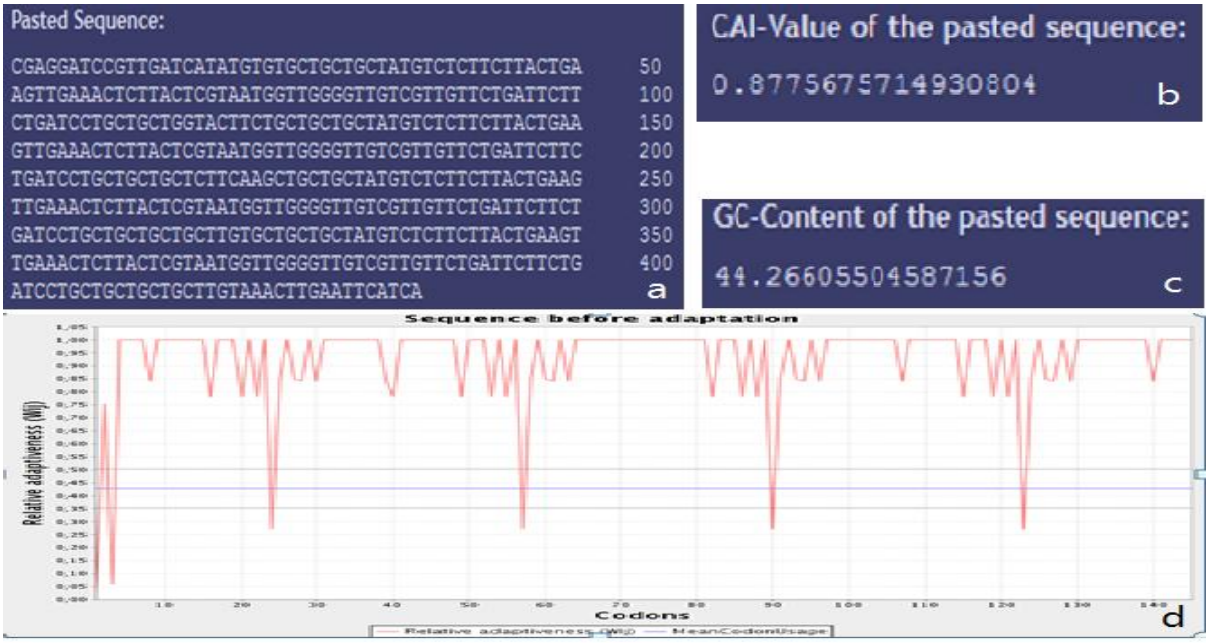

Fig. 2. The primary sequence of designed construct which is not adopted with usable codons in *B .subtilis.* a) Designed construct without adaptation with codons used in *B. subtili.* b) Value of adaptation without adaptation with codons used in *B. subtilis* is shown. c) GC-content of designed construct. d) Diagram of adaptation of this construct with native codons.

Fig. 3. The primary sequence of designed construct which is adopted with usable codons in *B .subtilis.* a) Designed construct with codons used in *B. subtilis.* b) GC-content of designed construct. c) Value of adaptation of this construct with codons used in *B. subtilis* is shown. d) Diagram of adaptation of this construct with codons used in *B. subtilis* is shown.

Six colonies were cultured on matrix plate and the plasmids containing gene were separated based on their size on agarose gel and analyzed by using restriction enzymes and PCR with primers M2e_F: 5'-aagcttgttgatcatatgtgcg-3' and M2e_R: 5'-gatccttaaagtttgcaagcag-3'.

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PCR was performed by using SmarTaq DNA Polymerase (Cinnagen, Iran) under following conditions : Stage 1 (1 cycle): 95°C for 4 min , Stage 2 (35cycles): 94°C for 30sec, 53°C for 30sec, 72°C for 45sec, Stage 3 (1 cycle): 72°C for 8min.

The recombinant shuttle vector was transferred into *B. subtilis* with optimized transformation buffer method (16). 100µl of transferred mixture were cultured on LB agar medium containing kanamycin (100µg/ml) (Sigma USA).

Results

Design and synthesis of construct

Selected sequence of *M2e* protein was from H9N2 influenza strain which was isolated in Iran in 2003 and its protein sequence is: MSLLTEVETLTRNGWGCRCSDSSDP. Five linkers: Linker1: VDHMCAAA, Linker2: AAGTSAAA, Linker3: AAALQAAA. Linker4: AAAACAAA and Linker5: AAAACKL were designed to fuse four M2e sequences that as shown in figure 1. Linkers 1 and 5 were inserted at the beginning and the end of the construct respectively and other 3 linkers were inserted between M2e sequences.

Codons of designed construct and its expression in *Bacillus* were investigated by software tools.

As indicated in figure 2, expression of this construct in *Bacillus* codons of construct were converted into codons which is usable for *B. subtilis* by using existing applications and then re-investigated by software that the results are shown in figure 3. As it is shown in this figure, the construct is designed with proper GC-content and the highest value of adaptation with codons used in *B. subtilis*. Expression of this cassette will be suitable

Cloning of M2e construct *in E. coli*

Double Restriction digestion of plasmid pUC57containing the construct by enzymes *BamHI* and *HindIII* was done and two bands of approximately 500bp for M2e construct and nearly 2.7kb for plasmid were obtained (Figure 4). The double digestion of expression shuttle vector pMR12 by the same enzymes showed up as a single band of 4.5kb on agarose gel

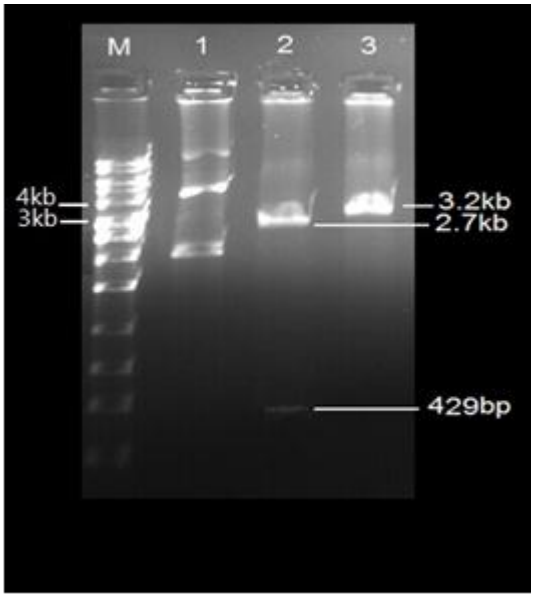

Fig. 4. Electrophoresis of single and double restriction digests of plasmid pUC57containing construct of M2e on 1% agarose gel: line M: 1kb ladder, line 1: plasmid pUC57, line 2: double digestion with *HindIII* and *BamHI*, line 3: single digestion with *HindIII*.

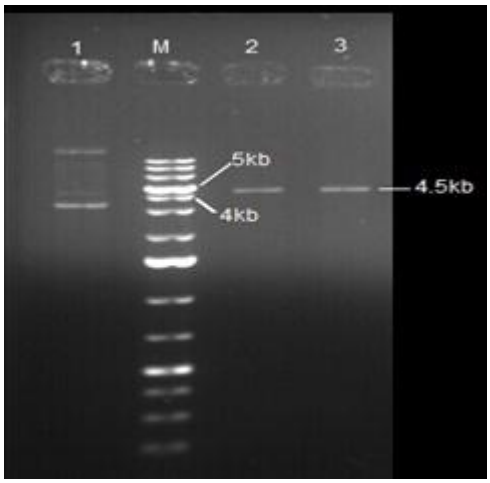

Fig. 5. Electrophoresis of digested pMR12 on 1% agarose gel: line 1: plasmid pMR12, line M: 1kb ladder ,line 2: enzymatic digestion with *HindIII* , line 3: enzymatic digestion with *BamHI*.

(Figure 5). Many colonies grew after transformation of ligation reaction and cultured on LB agar plate containing ampicillin. Six colonies were randomly selected and cultured and then plasmid was extracted from them; 3 samples had plasmids with our desired size.

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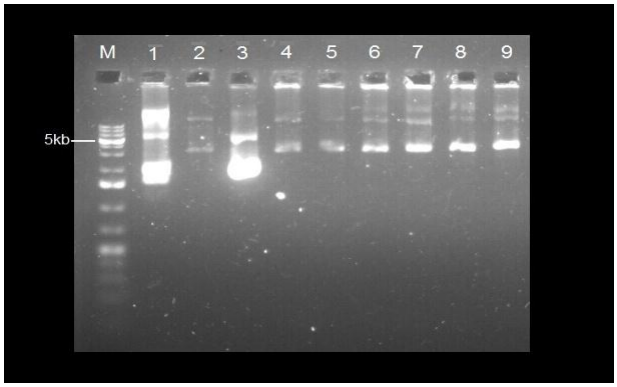

Fig. 6. Electrophoresis of extracted plasmid of 6 samples on 1% agarose gel. gel: line M: 1kb ladder, line 1: plasmid pUC57, line 2: empty plasmid pMR12 (negative control), line 3: plasmid pUC57 as a positive control used for determination of transformation efficiency. Line 4: Colony No. 1, line 5: Colony No. 2 .Line6: Colony No. 3, line7: Colony No. 4, line 8: Colony No. 5, line 9: Colony No. 6, sample No. 6 is 0/5 kb larger than rest.

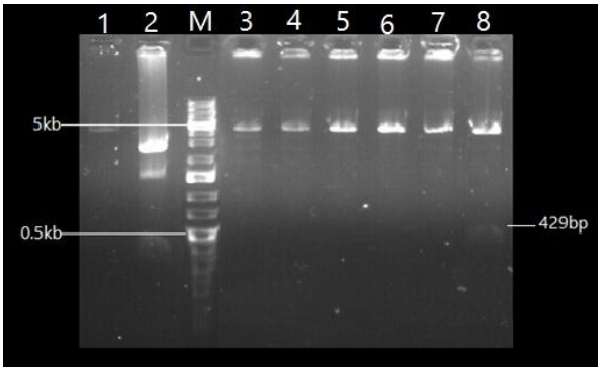

Fig. 7. Double digestion of extracted plasmid. Line 1: Double digestion of empty plasmid pMR12 (negative control), line 2: Double digestion of plasmid pUC57 (positive control), line M: 1kb marker, line 3*:* Double digestion of plasmid of Colony No. 1, line4: Double digestion of plasmid of colony No. 2. Line 5: Double digestion of plasmid of Colony No. 3, line 6: Double digestion of plasmid of Colony No. 4, line 7: Double digestion of plasmid of Colony No. 5, line 8: Double digestion of plasmid of Colony No. 6. Double digestion of plasmid pUC57 and plasmid of Colony No. 6 is showed up as bands of approximately 0.5kb.

PCR and digestion reaction by enzymes

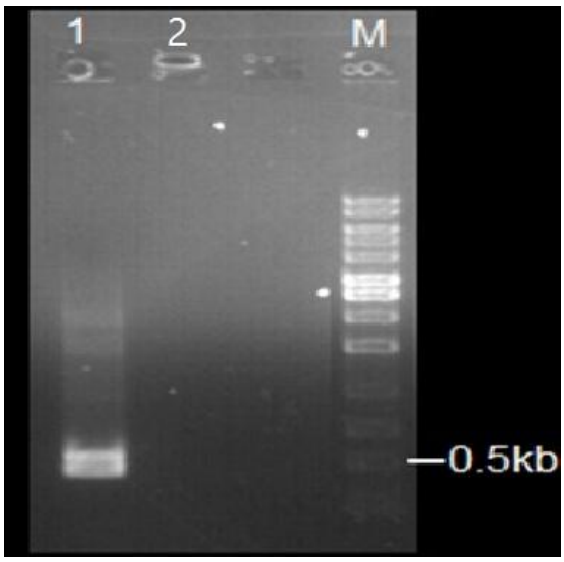

Fig. 8. Electrophoresis of PCR product. Line M: 1kb ladder, line 1: plasmid pUC57, line 2: PCR product on isolated plasmid of colony No. 6, line 3: PCR product on empty plasmid pMR12 (negative control).

BamHI and *HindIII* were done simultaneously on one sample and then the samples were electrophoresed (Figure 6-8). According to the results of the digestion reaction, considered gene sample was placed into the expression shuttle vector pMR12 correctly.

Cloning of gene construct of *M2e in B. subtilis*

With transformation of recombinant vector pMR12 into *B. Subtilis*, 9 colonies grew on media. Plasmid was extracted from all the samples and digested with a single enzyme. All the samples banded at position of 5kb which indicate 0.5kb fragment of gene. The existence of gene on all 9 samples was confirmed by using PCR with Specific primers. Sequencing of construct showed no changes suggesting the gene was placed correctly.

Discussion

Vaccination is one of the important way for animals and humans to provide significant protection against the influenza (17). Four main types of influenza vaccines include, an inactivated vaccine consists of intact influenza virus particle, HA

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or other proteins-based vaccines, live influenza vaccines and naked-DNA vaccines (5). Gene expression systems in a variety of ways under *in vitro* conditions for subunit vaccine preparations have been used (18). Current influenza vaccines are largely based on viral proteins. Genetic instability in HA and NA proteins is one of the main challenges in the production of recombinant subunit influenza vaccines. Ectodomain of matrix protein 2 of influenza, due to it's highly conservation in all subtypes of influenza A viruses, is an important antigen for development of a universal vaccine which able to play a role in protection against influenza (19). Fusion between 24 amino acid M2e peptide and the hepatitis B virus core leads to enhance the immunogenicity of the M2e protein and has provided mice ,complete protection to the challenge of influenza virus but the same vaccines has not effectively protected pigs (20). PapMV-CP has fused with M2e and causes an increase in the immunogenicity of the M2e protein (17). Ebrahimi *et al* have amplified M2e from influenza virus (H9N2) A/chicken/Iran/101/98 using RT-PCR with specific primers in 2009 and nucleotide sequence for M2e of influenza A was integrated into N-terminal fragment of *Mycobacterium tuberculosis* HSP70 (HSP70359-610) and pPICZaA was used as the expression vector (21). *Salmonella* flagell, outer membrane protein of *Neiseria meningitides* and B subunit of *cholera enterotoxin* along with M2e have adjuvant properties and stimulates the immune system (13, 22). Mirzaei and Rezaei have amplified M2e from influenza A virus (A/PUERTO RICO/8/34) using RT-PCR with specific primers in 2012 and after fusion with *ctx* gene of *Vibrio cholerae*, has cloned in pET28a vector (23). In 2013, Kim *et al* chose the consensus sequence of M2e (MSLLTEVETPTRNGWECKCSDSSD) and fused a 3xM2e tandem copy by 4 linkers and has cloned on pRSET vector. The use of the M2e peptide alone as a vaccine antigen was studied by this team also because peptides are unstable and not immunogenic. In this study, protein sequences of M2e of influenza A (MSLLTEVETLTRNGWGCRCSDSSDP) which isolated in Iran was used and fused a 4xM2e tandem copy by 5 linkers. This construct was cloned on expression shuttle vector pMR12 and finally transferred into *B. subtilis. Bacillus subtilis* has used as a good host for cloning and expression of genes because it is non-pathogenic and has ability to secrete a vast amount of high value proteins (25, 26). Because *M2e* gene does not undergo post-translational processing such as glycosylation, acetylation, and carboxylation, it can be cloned and expressed in the prokaryotic host that so far it has been cloned and expressed in *E. coli* (23, 24).This gene was cloned in *B. subtilis* during this study with the first.

Conclusion

This is the first report for successful cloning of a tandem repeat of 4 copies of M2e (4×M2e) along with linkers in *B. subtilis.*

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