Original Article

Construction of chimeric protein 3M2e.FliC and its immunoinformatics analyses and expression in *Bacillus subtilis*

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ABSTRACT

Introduction: Influenza A virus causes unpredictable epidemics and pandemics by creating antigenic variations. With the appearance of each new strain, rapid emergency countermeasures are taken against this new strain. Hence, designing an applicable and cross protective strategy to counter this virus is of great importance. To achieve this, choosing conserved antigenic regions in influenza virus proteins for making a universal vaccine is one of the best options. M2 channel in influenza virus membrane has a conserved sequence in ectodomain region called M2e. This region is the same between the majority of influenza A virus strains. But this peptide region is not a good stimulant for the immune system due to its short length. Immunoadjuvant property of *Salmonella Typhimurium* flagellin (FliC) has been previously shown to arouse immunity in the mucosal region. A combination of M2e and FliC was used in a construct in this study. **Methods:** To increase the possibility of immunugencity of this construct, *in silico* predictions of this fusion peptide construct were performed first. Three repeats of M2e gene and FliC sequence were cloned into pET28 vector and then were sub-cloned to pHT43. Finally, the construct was transformed into *Bacillus subtilis* by electroporation. After IPTG induction, the total cell protein and the supernatant protein were analyzed via protein analyses methods. **Results:** Based on *in silico* immunogenicity results, the designed recombinant peptide was deemed suitable to be used as a universal influenza vaccine candidate. The validity of pHT43.3M2e.FliC construct was confirmed by restriction map analysis. Western-blotting confirmed the presence of recombinant protein. **Conclusion:** The fusion peptide produced in this research, is the first step in designing a universal influenza vaccine which needs to be assessed in animal models alongside proper control groups in future studies.

KEYWORDS: Universal influenza vaccine, immunoinformatics, M2 Protein, *Bacillus subtilis*.

INTRODUCTION

Influenza A viruses are members of *Ortomyxoviridae* family and are capable of infecting a large variety of animals as well as humans. Hemagglutinin (HA) and neuraminidase (NA) are the most abundant proteins on the viral surface. Thus far, 18 different HAs (H1- H18) and 11 NAs (N1-N11) have been distinguished serologically and are currently used for nomenclature of the virus subtypes. Due to their segmented genome, zoonotic nature and occurrences of continuous mutations due to genome replication by a RNA polymerase with a high error-rate, influenza A viruses are able to increase or change their epidemic or pandemic features. This rapid evolution in influenza genome raises the need for updating vaccine formulations annually, in order to include new viral antigens [1, 2].

Current influenza vaccines have been made based on HA and NA antigens to elicit neutralizing antibodies and protection from influenza infection. However, owing to inherent variation of these target antigens, neutralizing antibodies induced by such vaccines would not be able to provide cross-reactivity against non-matching influenza strains. Thus, the development of a universal vaccine that provides cross-protection against all variant subtypes of influenza A virus has drawn more attention in recent decades [3, 4]. These include vaccines that induce antibodies directed against more conserved sequences like nucleoprotein (NP), HA2 region of the stalk domain, and matrix protein 2 ectodomains (M2e) [5, 6].

On the other hand, mass production of these virus-based influenza vaccines requires large-scale mammalian cell cultures or a large source of embryo eggs. These systems are costly and not rapid enough to match with a newly emerged influenza strain as witnessed in the 2009 H1N1 outbreak [7, 8]. Since

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1995, several studies have demonstrated that M2e vaccine candidates expressed in different hosts including baculovirus-expressed M2 [9], fusion proteins [10], and multiple antigenic peptides [11] could protect animals against challenges with homologous or heterologous viruses, as well as the heterosubtypic viruses.

M2 is an intriguing transmembrane protein in influenza virus structure which acts as a pH-gated proton channel in the virus envelope [12]. This homotetramer protein with 97 amino acids in each monomer comprises a short region in N-terminal ectodomain, a transmembrane domain and a long protein molecule which have ion channel activity and regulate vesicular and cytoplasmic pH within the virus-infected cells [13, 14]. M2 ectodomain which is known as M2e, includes 24 amino acids from N-terminal which has so far remained highly conserved. Moreover, another region of the N-terminal which includes 2 to 9 amino acids that is known as SLLTEVET epitope is conserved among all influenza A subtypes. Considering the existence of such conserved epitopes in M2, this region seems to be a reasonable choice for designing a universal vaccine [15]. Nevertheless, M2e conserved region is not large enough to trigger an effective immune response by itself. This problem has been solved by fusing different types of molecular adjuvants like HBc, ASP-1, CTA1-DD and bacterial flagellin [16].

Among Gram-positive bacteria, *Bacillus subtilis* has been developed as an attractive host for the expression of foreign proteins with pharmacological or immunological activities. In contrast to Gram-negative Escherichia coli, this bacterium contains no lipopolysaccharides (LPS) in its outer cell membrane. In addition, it has a natural high secretory capacity and exports proteins directly into the extracellular milieu which simplifies downstream purification and prevents the formation of inclusion bodies [17, 18].

In this study, a fusion gene of three tandem repeats of M2e was constructed along with Flagellin (FliC) as a molecular adjuvant. Moreover, the resulting mRNA and deduced protein and their stabilities alongside structural features were analyzed by bioinformatics software. This fusion protein was then expressed in *B. subtilis* successfully as a first step for production of a next generation universal influenza vaccine.

MATERIALS and METHODS

Bioinformatics analyses

To approve the recombinant protein production strategy, utilizing it as a vaccine and its considerable capacity of immunogenicity, it was important to examine all hypotheses in silico. In this regard, bioinformatics studies were conducted. The procedures were carried out in this order: codon optimization of sequences in *B. subtilis* with Optimizer Server software [19, 20], evaluating physicochemical characteristics in protparam software in ExPASy [21], evaluation of the secondary structure with Scratch proteomics online software [22], the tertiary structure of protein by phyre2 software [23], assessing the tertiary structure of protein by ramachandaran blot at UCLA-DOE LAB and QMEAN server [24, 25], prediction of humoral epitope sites[26, 27], prediction of MHC-I [28] and MHC-II epitopes [29] and the evaluation of allergenicity with Algpred online software [30]. The combination of our *in silico* studies culminated in the assessment of the predicted exposed epitopes.

Cloning and subcloning of 3M2e and FliC

The FliC and three M2e sequences in tandem (named 3M2e) segments were received as a gift from Dr. Mahdavi, Department of Immunology, Pasteur Institute of Iran. For ease of manipulation, these segments were cloned in two separate pET28a vectors. The FliC was inserted into *Hind*III and *Bam*HI sites in pET28a, and 3M2e were cloned with *Bam*HI site sequence in both ends in another pET28a vector. Subsequently, linear pET28a/FliC was prepared by digestion using *Bam*HI for insertion of 3M2e. Simultaneously, 3M2e was extracted from pET28a/3M2e by *Bam*HI enzyme to be inserted into the linearized pET28a/FliC. Employing this method, the 3M2e segment was cloned upstream of FliC segment in a fusion form. Correct orientation was confirmed by PCR technique. In PCR, we designed a pair of primers, the first half of forward primer was placed on the vector and second half on the beginning of 3M2e, the reverse primer was located in the midst of FliC gene. The correct orientation of 3M2e was verified by a 900 bp band of 3M2e gene fragment amplification by PCR.

Cloning of 3M2e.FliC chimeric segment in pHT43 expression vector and its transformation in E. coli and B .subtilis

Following preparation of chimeric 3M2e.FliC, it was cloned in pHT43 expression vector (MobiTec; Electronic Supplementary Material). To achieve this, 3M2e.FliC chimeric segment was amplified using PCR by Pfu enzyme. Two sets of primers were synthesized for PCR amplification of the two genes and cloning into pHT43. The sequence of forward primer was 5'-GGTCGCGGATCCAGTCTTC-3'. A reverse primer was replaced in the end of pET28a His-tag with a sequence of 5'-CGGATCTCAGTGGTGG-3' in addition to six complementing initial nucleotides of forward primer on pET28a and the rest on 3M2e.

Since 3M2e is consisted of 3 repeats of M2e, if the primer was made according to the beginning of M2e, there would be 3 connection possibilities, namely 3M2e at the beginning, at the middle of 3M2e and at the end. Therefore, connection to regions other than the beginning was prevented. The amplicon should have appeared as a 1634-bp band on electrophoresis gel. Considering the fact that the result of Pfu enzyme reaction was a blunt end segment, it was decided that the pHT43 vector be digested using a blunt-end enzyme (i.e. *Sma*I) which is present in the Multiple Cloning Site region. In this method, after ligation, it is necessary that the correct orientation of the inserted segment be confirmed. To achieve this, *Hind*III and *Aat*II were utilized to check whether the orientation of the segment was correct. The construction was confirmed by digestion with the above-mentioned enzymes leading to three fragments of 1800, 2100 and 5400 bp. Since the chosen host expression was a Gram-negative bacterium with a thick membrane which prevents heat-shock transformation method, we decided to use electroporation by an Eppendorf Multiporator device. A sample of 4 µl of the vector with 30 ng/µl concentration was inoculated with 18 µl competent B. subtilis, electroporated with 2 pulses each 5 ms duration with 2100 voltage. The bacteria were kept immediately in LB medium plus sorbitol monitol for 4 h in a 37° C incubator.

Gene expression in B. subtilis

The expression of recombinant protein encoded in pHT43 expression vector was carried by *B. subtilis* as follows. A colony was cultured overnight then renewed in OD 700 concentrations at 600 nm wavelength. Thereafter, IPTG was added in 0.5 M concentration. Since this expression system was capable of secreting recombinant protein in culture media, the

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presence of recombinant protein in the supernatant and the bacterial precipitations were evaluated. To check the presence of recombinant protein in cellular precipitation, ultra-sonication method was employed using ultrasound device (Heielscher, Ultrasound Technology UP200H) with cycle: 0.75, amplitude: 70% and 15 s period adjustment. The extraction of protein from cellular precipitation by ultra-sonication and from supernatant by protein precipitation was carried out. Protein precipitating with NaCl resulted in extraction of protein from the supernatant. The expected Mw (56 kDa) and integrity of the 3M2e.FliC were tested by SDS-PAGE (12.5%) and Western Blotting using Anti-His6 (2) mouse monoclonal antibody (Roche) and anti-mouse IgG (whole molecule) peroxidase conjugate (Sigma), detected by TMB substrate.

RESULTS

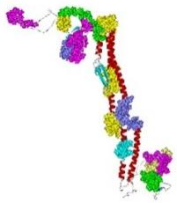
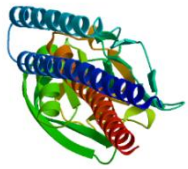
According to the bioinformatics evaluations, the recombinant protein was computed to have 56 kDa Mw and an acidic pH which the latter is a positive factor for purification. Thereafter, secondary structure of RNA was also examined, this was to ensure RNA resistance and its correct behavior in translation, the protein in question had considerable resistance with energy level of -635 Kcal Fig.1.



Fig.1. mRNA secondary structure prediction for 3M2e.FliC by calculating the minimum free energy of mRNA sequence. In this method, mRNA with the most negative value of free energy was considered the most structured and the most stable. Calculated free energy of 3M2e.FliC mRNA was equal to -634.99.

In designing the vaccine with recombinant peptide method, one of the most important aspects is to ensure that it is being exposed in epitope regions and not being an allergen. In the epitope regions, for obtaining the protein with the best modeling, d I-TASSER, PHYRE2, SWISS-MODEL online servers and modeler 9.15 offline programs were used and they were confirmed by QMEAN server Table 1.

Table 1. Comparison of predicted models based on QMEAN score, second model of SWISS-MODEL server with 0.564 credit score has the highest QMEAN score.

Model	Servers or software	Method	QMEAN Score
	Phyre2	Homology Modeling	0.325
	SWISS-MODEL	Homology Modeling	0.368
	SWISS-MODEL	Homology Modeling	0.564
	SWISS-MODEL	Homology Modeling	0.499
	Modeller 9.15	Homology Modeling	0.309

Based on the models credit score, a model from SWISS-MODEL server proved to be the best and was employed as recombinant peptide model Fig.2. After separate examinations on FliC and 3M2e and accepting their 3D constructions as being immunogen, the same examinations were performed on 3M2e.FliC fusion segment to ensure that the last construction's immunogenicity is concluded. This examination on fusion segment's 3D construction was to ensure that the linker is not detrimental to the proteins connection. This confirmed the correct folding and showed antigenic regions, whether exposed or not, are exposed despite the linker Fig. 2.

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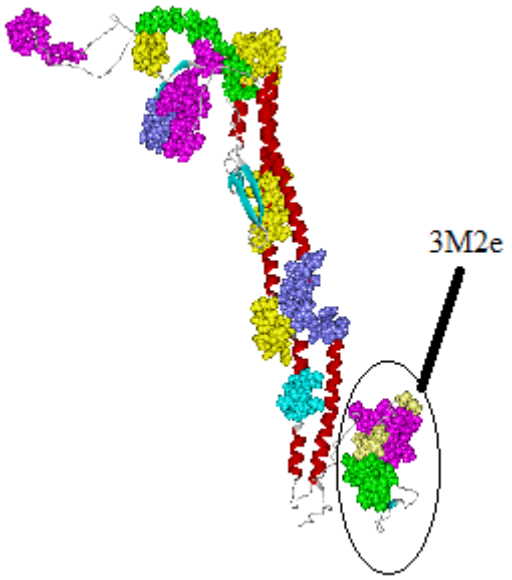

Fig. 2. Protein modeling of 3M2e.FliC fusion peptide by SWISS-MODEL server. Antigenic regions in 3M2e.FliC fusion peptide are exposed and the linker is not detrimental to 3D structure.

These *in silico* results indicated that the designed recombinant peptide is a suitable for consideration as a universal influenza vaccine candidate and may induce considerable immunogenicity. The results of PCR in designing fusion segment in pET28a body phase, demonstrated a 900-bp band which suggested the correct orientation of 3M2e in pET28a/FliC vector body. In the second PCR conducted on pET28a/3M2e.FliC construct, the amplification of 3M2e.FliC resulted a 1519-bp amplicon consisted of 3M2e, FliC and His-tag which was used for cloning in pHT43. Surveying correct orientation of fusion peptide in pHT43 vector with *Aat*II and *Hind*III enzymes resulted in three bands with about 2800, 2100, 5400 bps in colonies with correct orientated segment as shown in Fig. 3.

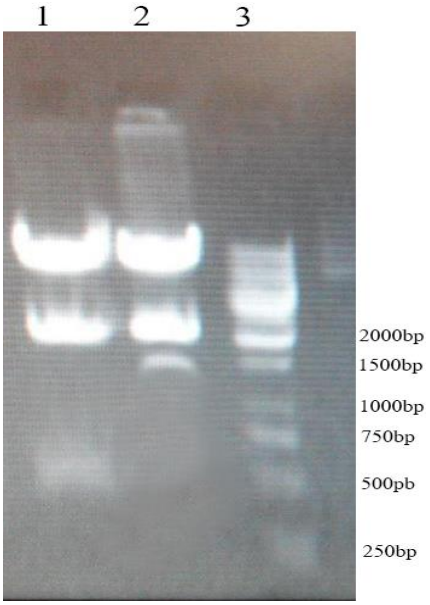

Fig. 3. Results of correct orientation surveying of fusion peptide in pHT43 vector with *Aat*II and *Hind*III enzymes. Lane1 shows incorrect orientation of 3M2e.FliC in pHT43 shuttle vector, lane 2 shows correct orientation and lane 3 is 1000-bp ladder.

Following expression, the extracted 3M2e.FliC was run by SDS-PAGE followed by Western Blotting and a band with expected size was detected which confirmed the presence of recombinant protein Fig. 4.

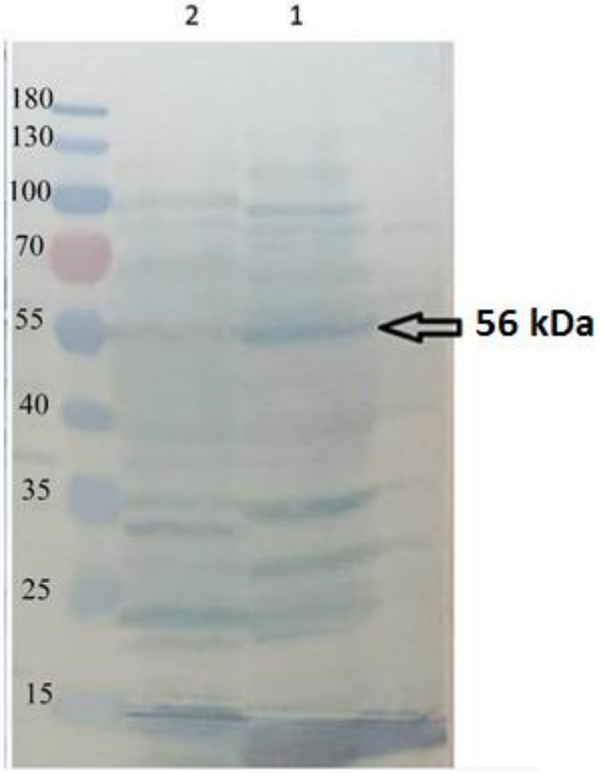

Fig. 4. Western-Blotting result indicating the recombinant protein band in lane 1 whereas it is not detected in lane 2 (T0).

DISCUSSION

Currently available influenza vaccines are based on NA and HA antigens which are vulnerable to mutations. Generally, influenza A pandemics emerge with new H or N antigens. The ideal solution to counter influenza pandemic is a vaccine that protects against all influenza A strains affecting humans; however such vaccines are expensive to produce. Among the components which has been used in universal influenza vaccines is M2e which compared to NA and HA antigens, has a highly conserved amino acid sequence. This conserved region is unchanging in almost all human strains and avian types, independent of the subtype. As a result, human type M2e cross-reacts with almost all avian M2e sequences. However, M2e is very short in size and is unable to cause immune responses. To overcome this issue, triple copies of M2e were fused to an adjuvant in this study. Recently, flagellin has gained prominence as an interesting adjuvant for use in human vaccines. For instance, bacterial flagellin as a TLR5 ligand, is a potent T-cell antigen and has potential as a vaccine adjuvant which unlike other TLR agonists, can stimulate Th1 and Th2 responses equally. Flagellin can also be used as an adjuvant mixed with the antigen but it is more frequently fused to a recombinant vaccine antigen [31, 32]. This adjuvant has other advantages, namely it is efficient at very low doses; it does not promote IgE response; it does not impair its adjuvant activity prior the immunity; its antigenic sequences can be inserted at the amino or carboxyl-termini or within the hypervariable region of the protein without any loss of flagellin signaling through TLR5 [31]. Moreover, it has been shown that flagellin induces no detectable toxicity in rabbits when administered intranasally [31].

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In a similar study, B. Turley *et al*. [16], have fused four copies of M2e epitope to the C-terminus of a full-length sequence of S. *typhimurium* fljB and they have demonstrated that four tandem copies of the M2e with fused flagellin adjuvant, induced a strong immune response in humans to the M2 ectodomain of influenza A virus. Moreover, they have demonstrated the importance of flagellin, as an adjuvant for a peptide antigen [16]. On the other hand, Gao *et al* [33], have demonstrated that 3 repeats of M2e plus an adjuvant could produce robust M2e specific antibodies and cellular immune responses [33]. A host with Generally Recognized as Safe (GRAS) status expression is of great importance in designing of vaccines. Choosing *B. subtilis* bacterium as a host with GRAS status and secretion ability was considered advantageous in this experiment [34, 35].

Unlike the next generation universal and recombinant influenza vaccines, the current influenza vaccines have limited immunogenicity due to their design which is based on antigens of just one subtype. This makes the production of present vaccines to be time-consuming and expensive. On the other hand, choosing universal antigen in influenza virus and designing a suitable immunogenicity strategy is the most important part of designing universal vaccines. In this study, the first step to design a next generation influenza vaccine was attempted, using a triple M2e peptide and flagellin as a molecular adjuvant. It is envisaged that this fusion construct could be evaluated as a universal vaccine and its immunogenicity potential be assessed in an animal model alongside proper control groups in future studies.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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