Comparative Immunology, Microbiology and Infectious Diseases 65 (2019) 76–80

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Immunological properties of the SLLTEVET epitope of Influenza A virus in multiple display on filamentous M13 phage

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ARTICLE INFO

Keywords:
Influenza A virus
M2e (2-9)
M13 phage
Immunization

ABSTRACT

Small peptides require large carriers to stimulate the humoral immune system. The filamentous phages, such as M13, have been proposed as vectors for expressing and carrying these peptides on their capsid surface. M2e 2-9 (SLLTEVET) residues of the transmembrane protein M2 of Influenza A virus are conserved and considered as a suitable target for immunization against a wide range of Influenza A virus strains. Here, M2e (2-9) sequence of Influenza A virus was fused to the N-terminus of the major coat protein gpVIII of M13 phage and was used to immunize broiler chickens. The results showed that the SLLTEVET peptide on the surface of M13 phage was expressed, the hybrid phage was immunogenic and produced specific antibodies against M2e (2-9) in broiler chickens.

1. Introduction

The filamentous phages (M13, fl, fd) offer several advantages over animal viruses for use as carrier for the display and enhance the immunogenicity foreign peptides [1]. Their capsid contains coat minor (III, VI, VII and IX) and major (VIII) proteins. Since there are around 2700 copies of pVIII per M13, this system allows the display of a large number of foreign antigenic peptides to be inserted near to the N-terminus of gpVIII, followed by a hybrid phage in which the major pVIII protein display the peptide epitope [2].

Influenza A virus which is responsible for illness in human and animals, has several important viral structural proteins, including Haemagglutinin (HA), Neuraminidase (NA), membrane protein (M), Nucleoprotein (NP), and nonstructural proteins [3]. The *M* gene encodes two conserved proteins, M1 (a capsid protein) and M2 (an ion-channel protein). The M2 protein is an integral membrane protein consisting of an ectodomain (M2e) at the N-terminus. M2e, which consists of 24 N-terminal residues, is remarkably conserved, and the N-terminal epitope SLLTEVET (residues 2-9) is particularly conserved across almost all studied subtypes of Influenza A viruses (99.3%) [4–6]. For the first time Lamb and Zebedee (1988) suggested that the production of monoclonal antibodies (14 C2) against the N-terminal of M2 could reduce the growth of Influenza A virus [7]. Also, Cho et al. (2015) solved the three-dimensional structure of M2e bound to an M2e-specific monoclonal antibody and showed that the conserved tryptophan residue at the center of the U-shaped structure of M2e is responsible for stabilizing its composition [8].

Due to the low induction of M2e-specific antibodies, which is resulted from the very weak immunogenicity of M2e in Influenza A virus infection [9,10], several studies have tried to enhance the immunogenicity of M2e through chemical or genetical conjugation of protein carriers. These protein carriers include, BSA, Keyhole Limpet Hemocyanin (KLH), GST, and *Neisseria meningitidis* outer membrane protein complex (OMPC), or delivery through Influenza virus-like particles (VLPs) [11–13], *Lactococcus lactis* [14], Multiple Antigen Peptide [15], T7 phage nanoparticles and phage Qβ [16,17], fusion with hepatitis B core antigen [18] and co-administration with different adjuvants [2,19].

We therefore attempted to fuse M2e (2-9) Influenza A virus to the N-terminal of the major coat protein pVIII to generate hybrid phages containing M2e 2-9-pVIII coat proteins and subsequently evaluating its immunogenicity in animal models.

2. Materials and methods

2.1. Bacterial strain and growth conditions

Escherichia coli strain TG1 was obtained with the Tomlinson I and J single-chain fragment variable) scFv(libraries from GeneService (Cambridge, UK). Bacteria were cultured in 2xTY broth or on TYE medium with or without 100 mg/L Ampicillin (Biobasic, Canada) at 37 °C.

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https://doi.org/10.1016/j.cimid.2019.05.004
Received 21 January 2019; Received in revised form 23 April 2019; Accepted 6 May 2019

2.2. Plasmid, gene synthesis and cloning

A chimeric gene containing the N-terminal epitope of M2e (SLLTEVET, 2-9), and the *gVIII* gene of phages M13 (from KM13) was designed. The corresponding gene was synthesized using (5′-CTTATACC ATGGCCAGCCTGCTGACCGAAGTGGAAACGGAGGGTGACGACCCCGC AAAAG -3′) and (5′-ACTCAGAATTCTTATCAGCTTGCTTTCGAGG-3′) oligonucleotides. PCR was performed in a 50 µL reaction mixture containing 2× Pre-mix (25 µL), 10 pmol of above primers, 5 µL of boiled DNA extracted from KM13 helper phage as template, 1.0 U of Phusion DNA polymerase (New England Biolabs, USA). Amplification program was set at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 45 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min.

Then, the amplified *M2e-gVIII* fusion gene and pIT2 vector were digested with *Nco*I and *Eco*RI restriction enzymes (Jena Bioscience, Germany), and consequently the *M2e-gVIII* gene was subcloned into pIT2 vector. The recombinant M2e (2-9)-gVIII pIT2 vector was then sequenced (Macrogen, Korea).

2.3. Generation, purification, and characterization of M2e-displaying M13 phage

M2e (2-9)-gVIII pIT2 vector was transformed into competent *E. coli* TG1 cells. For expression of hybrid phage, a single colony was incubated overnight in 50 mL of 2xTY broth containing Ampicillin (100 mg/L) and glucose (4% [w/v]) at 37 °C. The overnight culture was then diluted 1:100 into 250 mL of fresh 2xTY medium containing 100 mg/L Ampicillin and glucose (4% [w/v]) and was further grown at 37 °C with shaking. When the culture turbidity reached to an OD of 0.4 (600 nm), 1×10^{12} of KM13 helper phages was added. The sample was held at 37 °C without shaking for 60 min to allow phage infection and then centrifuged at 3200 *g* for 10 min. The bacteria were resuspended in 50 mL of 2xTY containing Ampicillin, Kanamycin (50 mg/L) and glucose (0.1% [w/v]) and grown overnight at 30 °C. After centrifugation, phages were precipitated from the culture supernatant (40 mL) by addition of 10 mL of a cold solution of 20% PolyEthylene Glycol 6000, 2.5 M NaCl and incubated for 1 h on ice. Viral particles were harvested by centrifugation at 3200 *g* for 30 min. The pellet was finally resuspended in 2 mL of PBS and centrifuged at 11,600 *g* for 10 min to remove any remaining bacterial debris.

2.4. Detection of expression of M2e peptide

2.4.1. Tricine-SDS-PAGE

In order to evaluate the expression of the recombinant fusion protein and its purity, the purified hybrid phages, HA2-pVIII M13 control phage (which was made exactly similar to M2e-pVIII M13 phage that carrying the conserved epitope of HA2 (GLFGAIAGF) portion of influenza virus [20]), and wild-type phage (KM13) were analyzed by Tricine-SDS-PAGE as described previously [21], with 5% stacking and 15% (w/v) separating gels. The gel was stained for proteins with Coomassie brilliant blue G-250 dye (Merck, Germany).

2.4.2. Western blotting

Hybrid phage and control phage (HA2-pVIII M13 phage) were run on an SDS-PAGE. Subsequently, these proteins were blotted onto a nitrocellulose membrane using a Mini-PROTEAN 3 apparatus (Bio-Rad). The membrane was blocked for 1 h at room temperature with 3% BSA/PBS, and then, for an extra hour in a fresh solution containing anti-M2e monoclonal scFv antibody which was prepared in our lab by three rounds of selection using antibody phage display technology from Thomlinson I&J libraries (manuscript in preparation). After three rounds of washes in PBS/0.05% Tween-20 (PBST), the membrane was incubated in anti-Poly Histidine Peroxidase (Sigma Aldrich, USA). Then colorimetric detection was performed using the 4-Chloro-1-naphthol substrate (Biobasic, Canada).

2.4.3. ELISA

An ELISA plate (Nunc, Denmark) was coated overnight with 100 µL (100 µg/mL) of hybrid phages, A/Chicken/Iran/SH-110/98 (H9N2) Influenza A virus (which was obtained from the Razi Vaccine and Serum Research Institute of Iran and prepared as we described previously [13]) as a positive control and control phage (HA2-pVIII M13 phage) as negative control at 4 °C and then blocked for 2 h with 3% BSA/PBS at room temperature. Then, anti-M2e monoclonal ScFv antibody was added to the antigen-coated plate and incubated for 2 h at 37 °C. After washing with PBST, the membrane was incubated with anti-Poly Histidine Peroxidase (Sigma Aldrich, USA) for 1 h at room temperature. After addition of TMB substrate, the plate was read at 450 nm.

2.5. Immunization with hybrid phage

To assess the immunogenicity of the hybrid phage, SPF chickens (7 Chickens / 2 groups, n = 14), were immunized (according to the local guidelines for the animal care) two times intramuscularly on days 8 and 15 with a total of approximately 1×10^{10} phage/200 µL of hybrid phage and PBS. The first immunization was done with complete Freund's adjuvant (Sigma) and subsequent dose was given without adjuvant. Blood samples were collected at the third week. The sera samples of each group were collected individually using centrifugation (1800 *g*, 10 min at room temperature). Then the activity of isolated serums was examined by ELISA assay using a commercially available M2e, human (SLLTEVETPIRNEWGCRCNDSSD) peptide as coating antigen (Genscript, USA) and anti-IgY Peroxidase (Genscript, USA) as the secondary antibody.

3. Results

3.1. Construction of M2e (2-9)-gVIII pIT2 vector

The gene coding for M2e-gVIII was amplified and cloned into pIT2 vector as shown in Fig. 1. Also, double digestion of this vector with *Nco*I and *Eco*RI restriction enzymes showed a nucleotide 164 bp as inserted fragment similar to amplified gene (Fig. 2). The construct was confirmed by sequencing.

3.2. Detection of M2e peptide expression on the surface of hybrid phage

3.2.1. Tricine-SDS-PAGE

The phage displaying epitope SLLTEVET, control, and wild-type phages were analyzed by Tricine-SDS-PAGE (Fig. 3). As this figure shows, two bands are observed for hybrid phage and control phage that indicate the major coat protein of these phages caring peptide whereas wild-type phage exhibits a single band.

3.2.2. Western blot and ELISA

To confirm the expression of SLLTEVET peptide by the hybrid

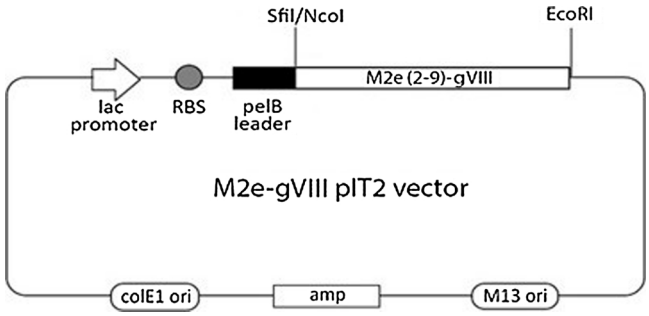

Fig. 1. Schematic representation of the constructed M2e (2-9)-gVIII pIT2 vector.

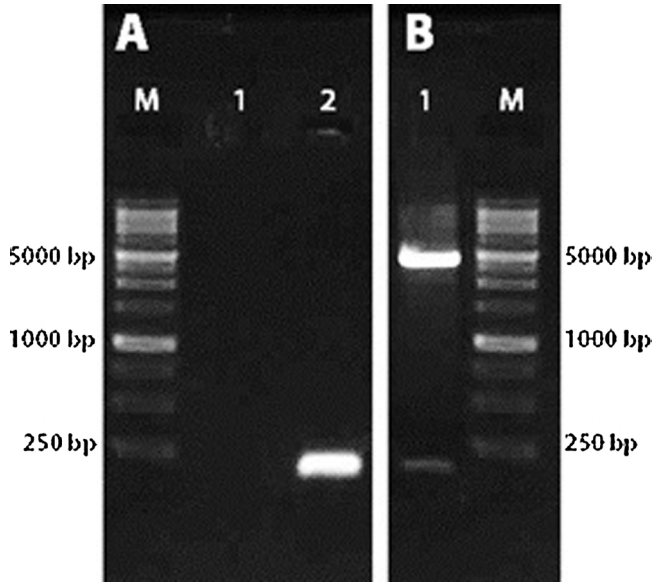

Fig. 2. PCR and restriction analysis of the constructed pIT2 vector on 1% (w/v) agarose gel. (A) PCR product with a molecular weight of 164 bp. Lane M: 1 kb DNA Ladder (Fermentas, Lithuania); Lane 1: Negative control; Lane2: amplified gene (M2e 2-9-gVIII). (B) Enzymatic digestion of M2e (2-9)-gVIII pIT2 vector with *Nco*I and *Eco*RI restriction enzymes. Lane M; 1 kb DNA Ladder (Fermentas, Lithuania); Lane 1: Digested M2e (2-9)-gVIII pIT2 vector.

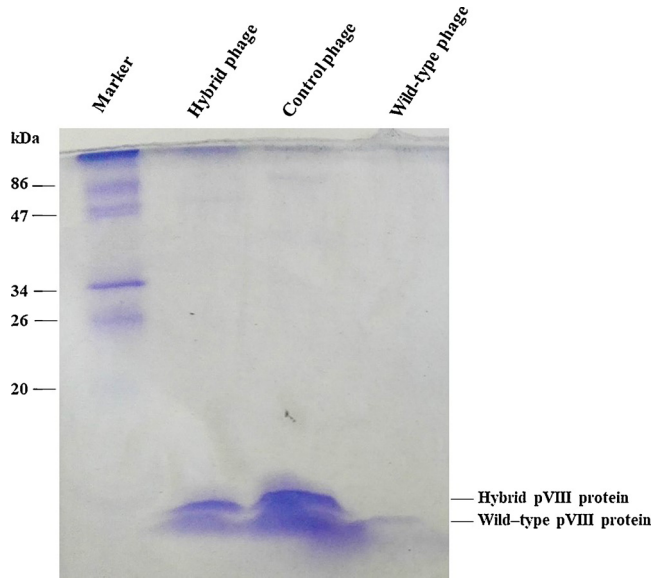

Fig. 3. Detection of hybrid phage, control phage and wild-type phage by Tricine-SDS-PAGE. From left to right: marker; 20–120 kDa Protein molecular weight (Bio Basic, Canada INC), Hybrid phage (M2e 2-9-pVIII phage), control phage (HA2 pVIII phage) and wild-type (KM13 phage). The molecular weight of wild–type pVIII protein is 5 kDa, and for hybrid and control pVIII proteins are ˜6 kDa.

phage, the interaction of an anti-M2e monoclonal scFv antibody with this phage was assessed by western blotting. As Fig. 4 shows the hybrid phage was detected by anti-M2e monoclonal scFv antibody while no band was detected in the control phage by western blot (Fig. 4).

Hybrid phages, H9N2 Influenza virus, and control phage were used for ELISA assay using anti-M2e monoclonal scFv antibody. ELISA results were comparable to those of the western blotting assay (Fig. 5).

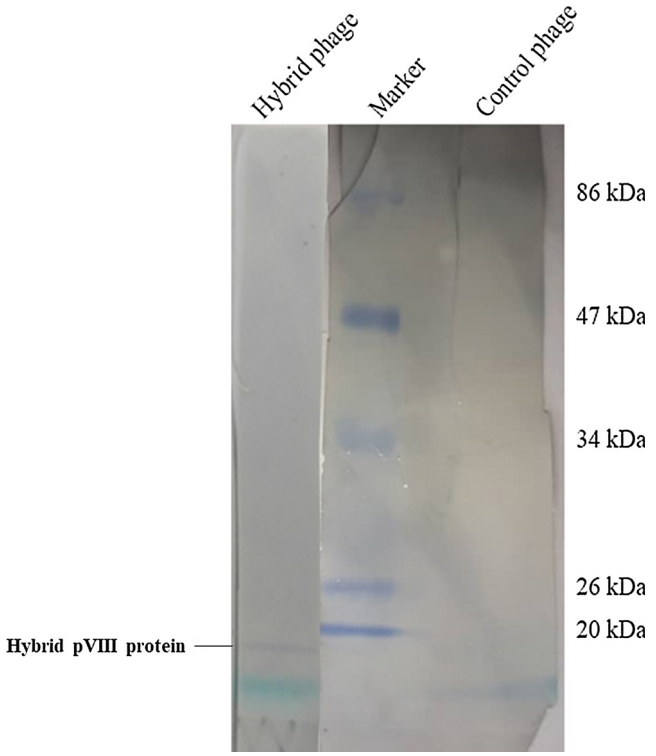

Fig. 4. Detection of the M2e peptide (SLLTEVET) using anti-M2e monoclonal scFv antibody by western blotting. Hybrid phage (M2e 2-9-pVIII phage) and control phage (HA2 pVIII phage) were separated on SDS-PAGE and transferred electrophoretically onto a nitrocellulose membrane. A horseradish peroxidase conjugated anti-Poly Histidine Peroxidas (Sigma) used as the secondary antibody. 20–120 kDa Protein molecular weight (Bio Basic, Canada INC).

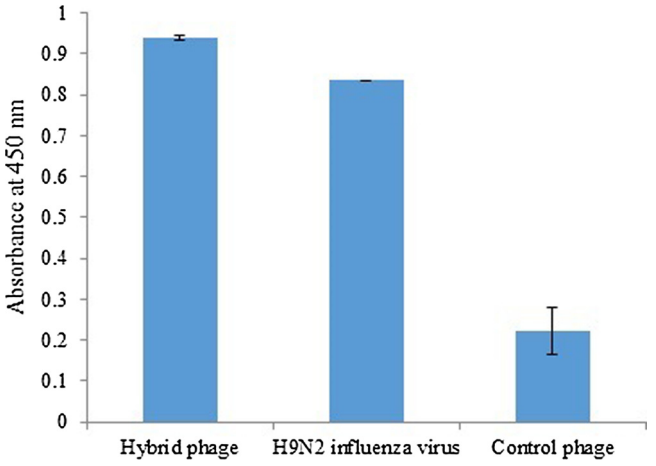

Fig. 5. ELISA assay for detection of M2e peptide (SLLTEVET) using anti-M2e monoclonal scFv antibody. The hybrid phage (M2e 2-9-pVIII phage), H9N2 influenza virus (positive control) and control phage (HA2 pVIII phage as negative control) were coated in ELISA plate. Anti-M2e monoclonal scFv antibody was applied as primary antibody and anti-Poly Histidine Peroxidas (Sigma) used as the secondary antibody. Reaction was read at 450 nm with TMB substrate.

3.3. Immunization

The chickens were immunized intramuscularly with hybrid phage and PBS. The sera were collected and tested with a commercial M2e peptide using ELISA assay. Results indicated high levels of antibody titer produced in chickens that received hybrid phage compared to the low levels of antibody produced in the control group (Fig. 6). This

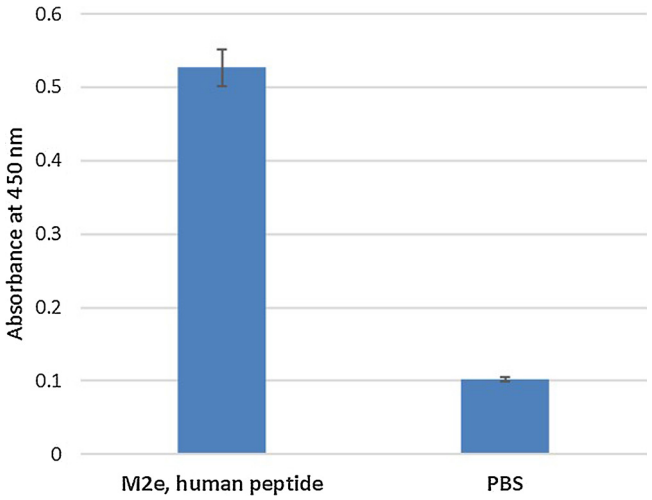

Fig. 6. ELISA assay for detection of specific anti-M2e (2-9) antibodies in broiler chicken serums. The M2e, human peptide and control (PBS) were used for coating of an ELISA plate. After adding diluted serums, anti-IgY horseradish peroxidase conjugated (Genscript) used as the secondary antibody. Reaction was read at 450 nm with TMB substrate.

indicates that the anti-M2e specific polyclonal antibody against SLLTEVET peptide was produced and therefore a successful immunization was achieved.

4. Discussion

The N-terminal 2-9 residues of M2e are almost completely conserved among Influenza A virus subtypes. These residues form an antigenic epitope that is suitable for immunization, and also the antibodies that are raised against this epitope can bind to infected MDCK cells [22]. Since SLLTEVET epitope is very small, it is needed to be coupled chemically or genetically to large carriers to enhance its immunogenicity. In current study, we genetically fused amino acids 2–9 of M2e to the N-terminal region of the major coat protein of M13 phage to increase the immunogenicity of this peptide. M13 phage has several advantages including physical stability, rapid and easy propagation in *E. coli* cells, and it is composed of 2700 copies of pVIII protein that could multiple the display of small peptides on the surface of the phage particle [23]. Several reports indicated that the size of heterologous peptide can affect the phage assembly and the copy number of recombinant pVIII in hybrid phage. For example it is described that insertion of more than six to eight amino acids at the N-terminus of pVIII decreased the viral assembly considerably due to interaction between pVIII and pVII proteins [24]. However, Lei Deng et al. reported that incorporation of full-length M2e of Influenza virus as a pVIII fusion into the filamentous phage of f88 was failed whereas decreasing length of M2e to15 amino acid M2e (2-16) led to 5–10% display of M2e (2-16)-pVIII. In our research we fused only eight amino acid of M2e (2–9) to the N-terminus of pVIII protein of M13 phage. The results of our Tricine-SDS-PAGE of hybrid phage exhibited an extra band near pVIII protein when compared with wild-type phage. Similar pattern was observed in previous studies with the phage displaying epitopes [25].

The expression of M2e (2–9) peptide on the surface of hybrid phage was confirmed by western blotting and ELISA using anti-M2e (2–9) monoclonal scFv antibody. The anti-M2e (2–9) scFv antibody reacted only with the hybrid pVIII protein but not with control phage (Fig. 4). These data indicated that the desired peptide has been successfully expressed on the surface of phage and specifically detected. We subsequently evaluated the immunogenicity of hybrid phage by injecting it intramuscularly into SPF chickens. At the end of immunization period, the chickens were bled to test their serums for IgY antibodies specific for M2e. To do this, an ELISA experiment was designed using a commercially available M2e, human peptide as the coating antigen. As the SLLTEVET residues of M2e were a part of this small peptide, the reactivity of serum antibodies of birds that immunized with hybrid phage with the above peptide in ELISA (Fig. 6) indicates that M2e peptide expressed on the surface of hybrid phage and led to production of specific antibodies in broiler chickens.

In summary, here we described the development of a filamentous hybrid phage expressing M2e (2–9) peptide on its surface that can be used to induce specific serum IgG antibodies against M2e protein of all Influenza A virus subtypes. This hybrid phage is a potential candidate for production of antibodies that could recognize a broad range of subtypes of Influenza A virus and probably for vaccination. Further studies are required to focus on utilizing its protection activity.

Competing interests

The authors declare that they have no conflict of interest.

Acknowledgments

We would like to thank the Research Council of Shahid Bahonar University of Kerman and Young Researchers Society of Shahid Bahonar University of Kerman for supporting this research. We are grateful to Dr. Elham Mohammadi and Dr. Maziar Jajarmy for their helpful advice.

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