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Enhancement of Immune Responses against Iranian Isolate of FMD-type O/IRN/1/2010 Based on VP1 and Human HSP70 Genes and Comparison with Conventional Vaccine

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ABSTRACT

***Background*:** Foot and mouth disease (FMD) is the causative agent and one of the most transmissible livestock diseases which cause important economic losses. The genome of *FMD* virus is a positive-sense, single-stranded RNA and it wrapped with 60 copies of 4 structural proteins, VP1, VP2, VP3 and VP4. The VP1 is a major immunogenic antigen with critical epitopes for inducing immune responses. The current vaccine, which successfully prevents disease, includes inactivated whole virus antigen. However, it is not without problems. The aim of this study is enhancement of immune responses against Iranian isolate of FMD-type O/IRN/1/2010 based on VP1 and human HSP70 fusion protein in *BALB/c* mice.
***Materials, Methods & Results*:** In this study FMD virus type O/IRN/2010 was isolated from vesicles of the infected cattle in Qoum, and serotyped as a new linage of type O (PanAsia-linage 2) in Iran. The isolated FMD *virus* was propagated on *IBRS2* cell line and whole RNA of the infected cells was extracted by commercial kit instruction. The extracted RNA was amplified using VP1 gene-specific primer pairs by means of one-step RT-PCR. The specific primer pair was designed by AllelID6 software. There are sequences of *Kpn I* and *BamH I* restriction enzymes and three overhanging nucleotides at the start of forward and reverse primers, respectively. The VP1 nucleotide sequence was deposit in Genbank-NCBI database under accession number JN 676146. The purified VP1 gene was sub-cloned into PTZ57R/T vector. Then digested VP1 gene by KpnI and BamHI enzymes was ligated in *pcDNA3.1*+ vector as a DNA vaccine. Also, the improved DNA immunization system was constructed using pcDNA3.1+ plasmid contains VP1 gene of Iranian isolate FMD virus type O/IRN/1/2010 and human HSP70 gene and expression of VP1-HSP70 fusion protein confirmed in *BHKT7* cell line by Guinea pig specific polyclonal antibody against FMD virus type O and conjugated rabbit anti Guinea pig antibody with HRP. Neutralizing antibody response, T cell proliferation assay and Interferon Gamma concentration against this cassette were evaluated on *BALB/c* mice in comparison of conventional vaccine and DNA vaccine which contains VP1 gene alone.
***Discussion*:** The inactivated conventional vaccine against FMD has some problems as: (1) the induced protection is short lasting; (2) it does not allow discrimination between infected and vaccinated animal; (3) production of FMD virus requires costly high containment facilities with permanent risk of virus escape; (4) difficulties can arise through insufficient chemical inactivation. Since the isolation of new FMD virus strains in different geographical locations and expression of VP1 gene using plasmid DNA based on HSP70 gene causes appropriate folding in viral protein, it can be used in emergency and control settings as a recombinant vaccine in the same area. In this study, the immune responses showed the improved DNA vaccine which contains VP1-Hsp70 fusion cassette, increasing of T cell proliferation response and IFN-gamma concentration significantly more than DNA vaccine which contains VP1 gene alone and conventional vaccine ($P < 0.05$). Also neutralizing antibody response for conventional vaccine was more significant than DNA vaccine and improved DNA vaccine ($P < 0.05$). However the improved DNA vaccine induced protective neutralizing antibody response. Since the Iranian isolate of FMDV type O/IRN/2010 caused outbreak in Iran, its neighborhood countries and Middle East, therefore the improved DNA vaccine based on VP1 and HSP70 genes is a good vaccine candidate against FMD virus type O/IRN/1/2010 and can use in this area.

Keywords: Foot and Mouth Disease virus, VP1 gene, HSP70, immune response, DNA vaccine, fusion.

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INTRODUCTION

FMD is a highly contagious disease of livestock, and causing acute illness, long-lasting persistent infection has been found in all cloven-hoofed animals such as: cattle, sheep, goats and others species [18]. FMD virus is a highly infective member of the *Picornaviridae* family and the genus *Aphthovirus* [4]. In recent years, one of the most important recombinant vaccines is DNA vaccine, allowing a safe and efficient alternative to conventional vaccination. Since the application of recombinant proteins by molecular techniques commonly have some limitations, chimeric protein was introduced. Chimeric proteins have found widespread application for the study of protein folding, structure stability and function [13]. The immune response induced by viral infection or conventional vaccination was not efficient for complete clearance of the virus. Recent studies showed that some DNA vaccines could elicit complete protection against the challenge of FMDV [4,16,17]. The DNA vaccines described as genetic immunization to elicit a protective immune response have been further improved by exploiting various gene delivery methods. Proteins of the HSP70/90 family are potent "natural" adjuvant that active the innate immune systems enhance antigen processing, presentation and stimulate cytokine production. Plasmids encoding fusion antigens with an N-terminal or C-terminal antigenic domains, HSP70-encoding sequence have been successfully used as DNA vaccines [3]. The aim of this study was enhancement of immune responses against Iranian isolate of FMD-type O/IRN/1/2010 based on VP1 and human HSP70 fusion protein in BALB/c mice and using this improved DNA vaccine in the Middle East in the next future.

MATERIALS AND METHODS

Virus

An infected cattle displaying FMD clinical symptoms was chosen to collect the epithelial cells of vesicles around Qoum-Iran in 2010. Pig kidney cells (IBRS2) were used to cultivate the suspension of infected epithelial cells in order to isolate the virus in Razi Vaccine and Serum Research Institute of Karaj, Iran. The inoculated cells showing considerable cytopathic effect (CPE) on the first passage were collected by freezing and thawing for preparation of FMDV antigen for the ELISA test and RNA extraction. Serotyping of the isolated FMDV antigens was done using polyclonal antibodies against the seven serotypes (FMD Diagnostic Kit)[1]. The total RNA was extracted directly from the supernatant of infected cells using RNeasy Mini Kit[2] according to the manufacturer's instructions.

Cloning of VP1 gene

The specific primer pair was designed by AlleID6 software package. There are *KpnI* and *BamHI* sequences and three overhanging nucleotides at the start of forward and reverse primers, respectively. The forward primer contains the kozak consensus sequence and start codon. The reverse primer contains two stop codons. The extracted RNA was reverse transcribed using the VP1 gene-specific primer pair and one-step RT-PCR kit'. The PCR product about 700 bp in size was cloned in *PTZ57R/T* vector[4] (Fermentas) and the cloned VP1 gene was confirmed by restriction enzyme digestion (*KpnI*[5] and *BamHI*[6]). The confirmed clone was sequenced using *PTZ57R/T* universal primer and the nucleotide sequence data was deposited in Gen Bank database. The VP1 gene was digested from *PTZ57R/T* vector by *KpnI* and *BamHI* enzymes and used to construct fusion cassette. Also it cloned in *pcDNA3.1*+ vector[7] as a DNA vaccine against FMD virus type O/IRN/1/2010 [6, 10].

Cloning of HSP70 gene

The cloned E7-HSP70 genes in pcDNA3.1+ vector was constructed by Dr. H. Soleimanjahi, papilloma virus E7 gene was cloned between *KpnI* and *BamHI*, also human HSP70 gene was cloned into the unique *BamHI* and *XhoI* cloning sites [13]. The cloned human HSP70 gene in pcDNA3.1+ vector was confirmed using restriction enzymes digestion (*BamHI* and *XhoI*). The clone was digested by *KpnI* and *BamHI* and E7 gene was separated, therefore pcDNA3.1^{+} vector which contained human HSP70 gene was applied as backbone to construct improved DNA vaccine with VP1-HSP70 genes (fusion).

Construction of fusion cassette as improved DNA vaccine

The digested FMD virus type O/IRN/1/2010 VP1 gene by *KpnI* and *BamHI* enzymes was inserted into the pcDNA3.1+ vector which contain human HSP70 gene to construct the VP1–HSP70 fusion cassette. The fusion cassette was confirmed using restric-

tion enzymes digestion (*KpnI* and *XhoI*). The fusion cassette was called improved DNA vaccine against FMD virus type O/IRN/1/2010.

Transfection and Protein expression

Four hundred thousand *BHKT7* cells were seeded onto cover-slips on six-well plates and incubated at 37°C in a CO_2 incubator until the cells were 50-70% confluent. The following day, 10 µg of plasmid DNA (*pcDNA*3.1+ vector which contain Vp1-HSP70 genes and *pcDNA3.1*+ vector without any additional gene) in 100 µL of DMEM without serum was mixed with 7 µL of Lipofectamine™ reagent[8] in 100 µL of DMEM[9] without serum. The mixture was then incubated at room temperature for at least 30 min before it was diluted into 800 µL reduced-serum DMEM and added to the cells. After incubation for 5 h at 37°C in a humidified incubator, 1 mL of medium containing 5% fetal calf serum[10] was added to each well. After 48 h the transfected cells were washed by PBS buffer and harvested by lysis buffer containing PMCSF[11] (protease inhibitor). The harvested cells centrifuged 16000 g, 4°C in 20 min, and supernatant was used for SDS-PAGE and western blotting analysis. The specific band of VP1-HSP70 fusion protein was detected by Guinea pig specific polyclonal antibody against FMD virus type O and conjugated rabbit anti Guinea pig antibody with HRP as second antibody[1].

Vaccination

Twenty five *BALB/c* female mice (4-5 weeks old) were provided by the RAZI Vaccine and Serum Research Institute (Karaj, Iran). The *BALB/c* mice were randomly divided into five groups. Groups 1 and 2 were control groups inoculated with 100 µL PBS and 100 µg of plasmid *pcDNA3.1*+, respectively. Animals in groups 3 and, 4 were administrated with 100 µg of plasmid *pcDNA3.1*-VP1 (DNA vaccine) and fusion plasmid (improved DNA vaccine) which contain Vp1-HSP70 genes, respectively. The final group 5 was injected by the conventional vaccine (inactivated by BEI) against FMDV type O/2010. All of the first four groups were boosted with the same inoculation at 2 weeks intervals two times and the final group was boosted after 21 days. The route of administration for all of the animals was subcutaneous. All groups of mice were bled 10 days after the last injection. The sera were separated from the blood samples and the complement factors of the sera were inactivated at 56°C for 30 min. Then sera were tested for the presence of neutralizing antibodies against FMDV type O by serum neutralization test [9,14].

Preparation spleen cells and T-lymphocytes proliferation assay

The splenic lymphocytes were removed and cultured using a T-lymphocytes proliferation assay with MTT (3-4,5-dimethylthiazol- 2,5-diphenyltetrazolium-bromide). Spleens of vaccinated mice were removed aseptically 10 days after last injection. The splenic lymphocyte suspensions were prepared and incubated in 96-well plates at 5×10^4 cells/well by RPMI 1640 plus 10 % of fetal calf serum (FCS) at 37°C in a 5 % CO_2. The cells were stimulated with 50 µL of Phytohemagglutinin (50 µg/mL) (positive control), 2.5 µg/mL of 146S antigen of FMDV type O IRN/1/ 2010 (specific antigen stimulation) and no antigen (negative control), in triplicates. After 48 h the MTT assay was performed using a Cell Proliferation Kit[12] according to manufacturer's instructions and absorbance was measured at 540 nm and stimulation index (SI) was calculated [9].

Cytokines assay

For measurement of IFN-γ splenic lymphocytes were incubated as described above for the proliferation assay. After 48 h, supernatants were collected and different dilutions were assayed in duplicate using commercial ELISA kits for IFN-γ (eBioscience Mouse IFN-g kit[13]).

Statistical analysis

Statistical analysis was done by analysis of variance (ANOVA-One way) followed by Duncan's multiple range test by SPSS16 software. *P* values of less than 0.05 were regarded as significant.

RESULTS

Serotyping of the isolated FMDV antigen was showed serotype O by ELISA technique. The VP1 nucleotide sequence data of FMDV type O/IRN/2010 was deposited in Gen Bank database under the accession number JN 676146. FMD virus type O/IRN/1/2010 VP1 gene caused a DNA band about 700 bp after digestion of *PTZ57R/T* vector by *KpnI* and *BamHI* enzymes (Figure 1). Plasmid of *pcDNA3.1*+ which contained human HSP70 gene was also extracted from agarose gel after digestion of E7 by the same enzymes.

Ligation of VP1 gene into the *pcDNA3.1*+ vector made a cassette of VP1-HSP70 fusion. Double digestion of fusion cassette by *KpnI* and *XhoI* enzymes to make a band with 2500 bp which include VP1 (700 bp) and HSP70 (1800 bp) genes in Figure 2 B, also the result of PCR reaction by a pair specific primers for VP1 was made a band with 700 bp which was VP1 gene amplification (Figure 2 A). Both of these reaction confirmed the ligation of VP1-HSP70 fusion in *pcDNA3.1*+ vector.

To evaluation of VP1-HSP70 fusion protein, the lyses of transfected BHKT7 cells with the fusion cassette and *pcDNA3.1*+ vector alone after 48 h were analyzed using SDS-PAGE and western blotting and observed the specific fusion protein band, about 90 kDa (Figures 3 and 4).

Serum Neutralization Test

The anti FMDV type O/IRN/1/2010 sera titration of the vaccinated mice is shown in Table 1 at 10 days after last vaccination. The mice were immunized subcutaneously using plasmid which express FMDV VP1-Hsp70 fusion protein showed protective neutralizing antibody titer against FMDV type O/IRN/1/2010 and significant differences compared with the negative control groups (the groups which were immunized by PBs, *pcDNA3.1*+) ($P < 0.05$). However the responses of vaccinated mice with PBS and *pcDNA3.1*+ vector were not protective. Third group was vaccinated by vector which expresses FMD type O/IRN/1/2010 VP1 showed significant differences compared with the negative control groups for neutralizing antibody titration, but not protective response. Also neutralizing antibodies response for conventional vaccine was more significant than DNA vaccine ($P < 0.05$).

T-Lymphocytes proliferation and cytokines assay

The results of the MTT assay and IFN-γ concentrations in five groups of mice are shown in Table 1. The SI values and IFN-γ concentrations of the vaccinated mice groups with plasmid which contains VP1 gene, VP1-Hsp70 fusion cassette and conventional vaccine were significantly higher than control groups (PBS and *pcDNA3.1*+ vector) at 10 days after the last vaccination ($P < 0.05$). Also these two items for fourth group was more than third and fifth groups.

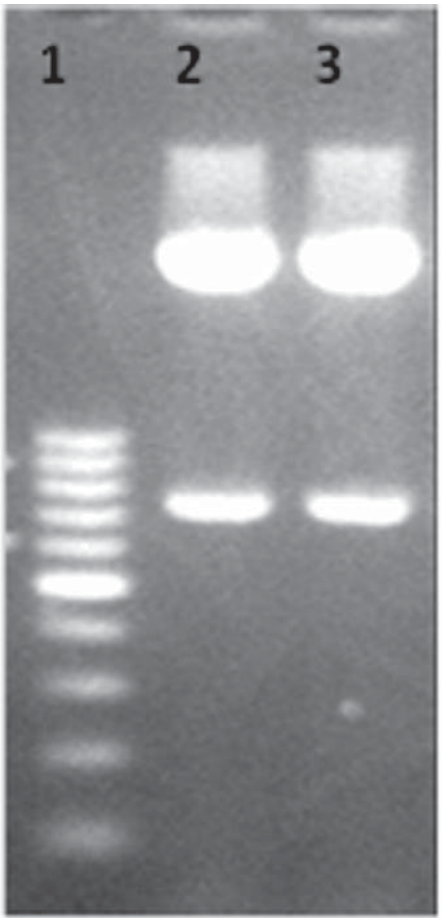

Figure 1. Digested *PTZ57R/T* vector caused VP1 band (about 700 bp). Lane 1:DNA Ladder (Fermentase SM1143); Lanes 2 and 3: digested *PTZ57R/T* vector.

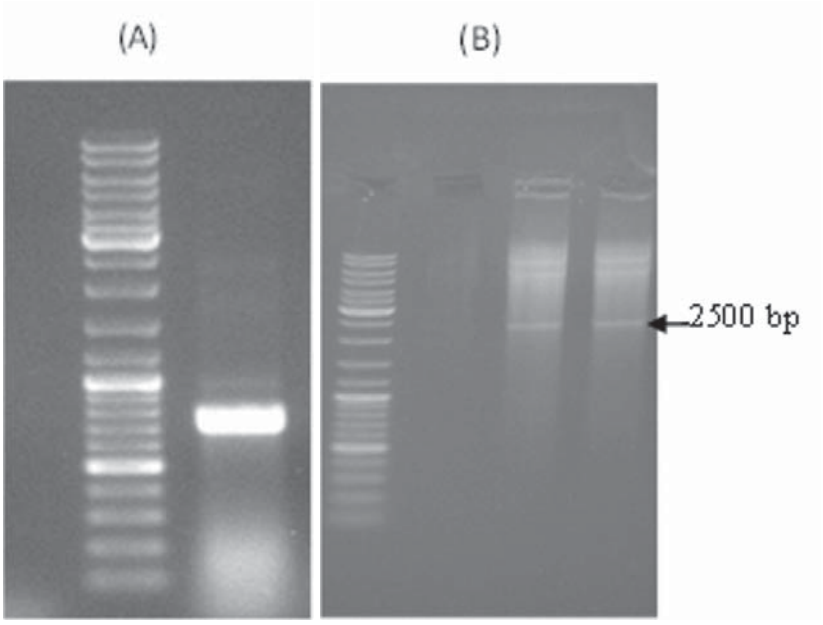

Figure 2. (A) Amplification of VP1 gene by PCR reaction to confirm ligation. Lane 1: DNA Ladder (SM0333); Lane 2: VP1 gene (about 700 bp); (B) Digestion of VP1-HSP70 fusion by KpnI and XhoI enzymes.

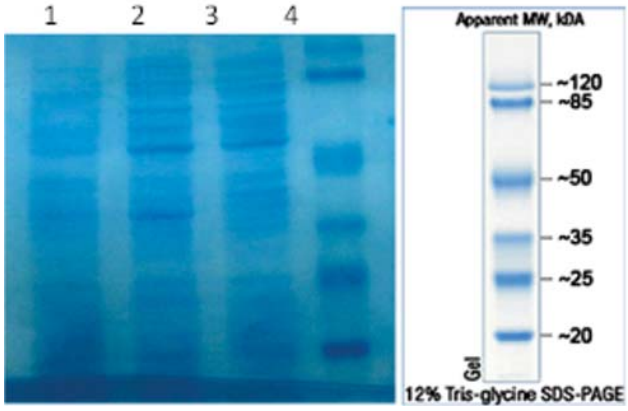

Figure 3. SDS-PAGE electrophoresis analysis of transfected BHKT7 cell lyses. Lane 1: lyses of the transfected cell with pcDNA3.1+ vector; Lane 2: lyses of the transfected cell with VP1-HSP70 fusion; Lane 3: lyses of untransfected BHKT7 cell; Lane 4: Protein Marker (fermentase, SM0441).

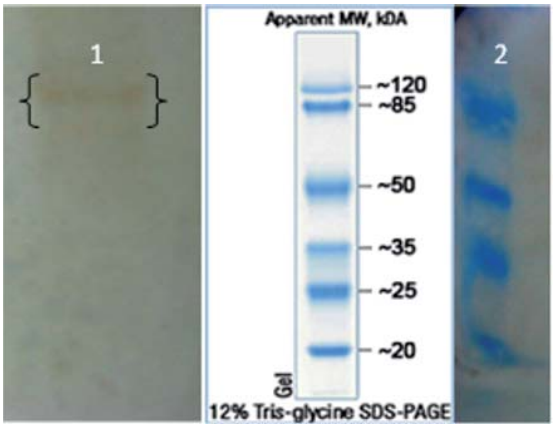

Figure 4. The specific band of VP1-HSP70 fusion protein in western blotting analysis (Lane 1), Protein Marker, fermentase, SM0441 (Lane 2).

Table 1. Antiserum titration, T cell proliferation assay and IFN-γ concentrations of the vaccinated mice against FMDV type O/IRN/1/2010 ten days after last vaccination.

Group No.	Type of vaccine	Mean of Antiserum titration± SE	MTT assay (SI±SD)	IFN-γ±SD (pg/mL)
1	PBS	≤0.6± 0.0745 (Non protective)	0.98±0.051	3.844±0.58
2	pcDNA3.1+	≤0.6± 0.0745 (Non protective)	1.03±0.020	8.60±4.08
3	DNA vaccine (pcDNA3.1+VP1)	1.1± 0.0745 (Non protective)	1.23±0.123	619.76±144.28
5	Improved DNA vaccine (pcDNA3.1+VP1-Hsp70)	1.3± 0.0745 (protective)	1.44±0.131	1098±233.61
6	Conventional vaccine	1.9± 0.074 (protective)	1.26±0.095	464.27±355

DISCUSSION

FMD virus is still an important pathogen of worldwide and it frequently escapes from endemic to non-endemic areas. The development of new recombinant vaccines is still an objective of immense practical importance [1 and 11]. FMDV type O has five antigenic sites which three of them are located in Vp1 sequence; therefore VP1 is the most immunogenic peptide for FMD virus. As this fact, VP1 gene of FMD type O/IRN/1/2010 was used for preparation DNA vaccine in this research and could stimulate neutralizing antibody, spleen T lymphocyte and IFN-γ more than negative control groups.

The conventional vaccine against FMD virus is a chemically inactivated whole virus preparation with adjuvant. This vaccine, like natural infection, induces a rapid neutralizing antibody response, but memory T cell responses are usually weak and therefore are unlikely significant contribution to the observed memory response [12]. While the T cell response is not known to directly affect the outcome of infection, more effective T cell responses might support the induction of sterile immunity and extend the duration of the protective immune response [2]. HSPs are important modulators of antigen presentation, T-lymphocyte activation, cytokine production, and NK cell killing, placing them in a unique position of contributing to both intracellular and extracellular responses to a physiological stress [5,7,15]. Hsp70 can improve the delivery of FMDV antigen to the MHC II pathway of antigen presenting cells resulting in enhanced T cell stimulation [8]. According to this research, T cell proliferation and IFN-gamma concentration of improved DNA vaccine were increased more than negative control, conventional vaccine groups and DNA vaccine alone. The results can confirm which VP1-HSP70 fusion cassette can stimulate neutralizing antibody and T cell responses and make protective response. Therefore we can suggest HSP70 gene as a fusion with the virus genes for increasing the efficacy of DNA vaccine against *FMD* virus in next future. Also using the fusion cassette of VP1-HSP70 and inactivated conventional vaccine as a novel prim-boost vaccine is another suggestion.

SOURCES AND MANUFACTURERS

[1]FMD Diagnostic Kit - Pirbright, UK.

[2]RNeasy Mini Kit - Qiagen, Hilden, Germany.

[3]One-step RT-PCR kit[3] - Bioneer, Korea.

[4]PTZ57R/T Vector - Fermentas, Lithuaniav.

[5]*KpnI* Restriction Enzyme - Fermentas, Lithuaniav.

[6]*BamHI* Restriction Enzyme - Fermentas, Lithuaniav.

[7]*pcDNA3.1*+ vector - *Invitrogen*, USA.

[8]Lipofectamine TM reagent Invitrogen, USA.

[9]DMEM, Gibco, USA.

[10]Fetal Calf serum, Gibco, USA.

[11]PMCSF, Sigma, Germany.

[12]Cell Proliferation Kit -MTT, Roche, UK.

[13]Mouse IFN-γ ELISA kit- eBioscience, USA.

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***Ethical approval*.** The procedure was approved by the medical ethic Committee of Health Ministry of Iran in 2008.

***Declaration of interest*.** The authors report no convicts of interest. The authors alone are responsible for the content and writing of the paper.

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