

***Original Article***

## **Evaluation of cytokine mRNA expression in vaccinated guinea pigs with foot-and-mouth disease type O inactivated vaccine**

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### **ABSTRACT**

Foot-and-mouth disease (FMD) is a severely contagious viral disease that mainly affects cloven-hoofed livestock and wildlife. This study quantifies the cytokines mRNA expression of vaccinated guinea pigs with FMD type O inactivated vaccine. Blood samples were collected from eight guinea pigs at 7 and 28 days after the first vaccination. Extracted mRNAs were reverse-transcribed into cDNA and analyzed for quantification of IFN- $\gamma$ , TNF- $\alpha$  and IL-10 expression using relative real-time PCR assay. Our results showed that all of the genes were upregulated. The expression of TNF- $\alpha$  and IL-10 genes significantly increased ( $P < 0.05$ ) in day 28th in comparison to the day 7th post the first vaccination. It can be concluded that the vaccine induced immune responses by increasing expression of the cytokines. Therefore, effects of DNA vaccines on immune system also may be evaluated using these genes.

**Keywords:** Cytokine, mRNA expression, FMD, Guinea pig, Real-time PCR

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### **INTRODUCTION**

Foot-and-mouth disease virus (FMDV), a member of the *aphthovirus* genus within the *picornaviridae* family, is the causative agent of a highly contagious disease affecting cloven-hoofed animals (Alexandersen et al., 2003; Suttmoller et al., 2003). This disease causes considerable economic losses in livestock productivity. Seven distinct serotypes of FMDV have been identified, namely O, A, C, South African Territories (SAT) 1, SAT 2, SAT 3 and Asia 1, and over 60 strains

within these serotypes. Serotype O is the most common serotype in over the world (Grubman and Baxt, 2004).

Foot-and-mouth disease (FMD) can be controlled by slaughter of infected and exposed herds, or by vaccination. In countries where the disease has not been eradicated, vaccination plays a critical role in its control. One of vaccines performances is stimulating expression of some immune system genes, which are called cytokines. Genes encoding of T-helper 1 (Th1) and Th2 cytokines are two major types of genes that contribute in the immune system (Saito et al., 2010). Cytokines are proteins which secreted by innate and

acquired immune systems cells in response to a stimulus and modulate many functions of target cells (Elenkov et al., 2005). The actions of cytokines are complex so that the same cytokine can have different effects on a cell, depending to the state of the cell. For example, there are several known cytokines that have both stimulating and suppressing actions on lymphocyte cells and immune response (Nadeem et al., 2004). In previous studies, it has been elucidated that the levels of some cytokines were induced in immune response to FMDV in cattle (Zhang et al 2006) and water buffalo (*Bubalus bubalis*) (Mingala et al., 2009). Recently, DNA vaccines have developed against FMDV which requires performance evaluation. Measurement of cytokine mRNA expression in vaccinated laboratory animals such as guinea pig provides useful information about these vaccines. Therefore, the effects of DNA vaccines on immune system may be investigated using the cytokines genes. Guinea pig is the most susceptible laboratory animal to FMDV. Previously guinea pig model was used for analysis immune responses of FMD type A87/IRN inactivated vaccine in Iran (Sedeh et al., 2008). Nevertheless little information is available on the quantification of cytokines expression against FMD inactivated vaccine in guinea pig. Hence, the current study was performed to evaluate mRNA expression of interferon gamma (IFN- $\gamma$ ), tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin-10 (IL-10) cytokines against FMD inactivated vaccine using quantitative real-time PCR technique in guinea pig. Other objective of the research was introduced guinea pig as an animal model and cytokines induced in response to FMD inactivated vaccine in order to assess of DNA vaccines effects in future studies.

## MATERIALS AND METHODS

**Experimental animals, vaccination and blood collection.** Eight pathogen-free out-bred Hartley strain male guinea pigs (400–500 g) in 2 groups (Four for each group) were used in this study. They were housed in two cages, fed commercial guinea pig chow

and maintained in a temperature and humidity conditioned environment on a 12 h light/dark cycle. The animals rested for 10 days under the conditions. Four of the animals were used as treatment (vaccinated animals), while the other half was used as control (non-vaccinated animals). The treatment animals were vaccinated via intradermal injection in footpad with 0.5 ml of FMD type O inactivated vaccine for two times at day 0 and subsequently 14 days after the first vaccination. This vaccine was produced by Razi vaccine and serum research institute of Iran. The control animals were injected only with 0.5 ml of phosphate-buffered saline (PBS). Blood samples (0.5-1 ml) from all the animals were collected from the saphenous vein into EDTA-containing micro tubes at 7 and 28 days after the first vaccination and stored at -70 °C until analyzed.

**Design of primer sets.** The sequences of IFN- $\gamma$ , TNF- $\alpha$ , IL-10 and the  $\beta$ -actin (as an internal control) genes have been assigned the accession numbers NM\_001172874, U77036, AF097510 and NM\_001172909, respectively, in the GenBank database. Real-time primers for these genes were designed using Primer Premier v. 5.00 software (Premier Biosoft. International, Palo Alto, CA, USA) and are described in Table 1.

**Total RNA isolation.** Total RNA from each sample was isolated using the Column RNA isolation kit (Denazist Asia, Iran) according to manufacturer's instruction. The RNA preparations were treated with RNase-free DNase I to eliminate possible contaminating DNA, quantified using NanoDrop™ 1000 spectrophotometer (Thermo-Scientific, USA) and stored at -80°C. RNA purity was confirmed by OD<sub>260</sub>/OD<sub>280</sub> absorption ratio determination.

**cDNA synthesis.** Extracted mRNAs were reverse-transcribed into cDNA using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Germany). It was performed in a 20  $\mu$ l final volume including 2  $\mu$ l of sample containing 90 ng extracted RNA, 1  $\mu$ l of 100  $\mu$ M oligo-dT and 9  $\mu$ l of DEPC-treated water. The mixture was heated at 65 °C for 5

minutes and immediately placed on ice. Then 4  $\mu$ l of 5X Reaction Buffer, 1  $\mu$ l of 20 U of RiboLock RNase Inhibitor, 2  $\mu$ l of 10 mM dNTPs and 1  $\mu$ l of 200 U of MM-MuLV Reverse Transcriptase were added to the mixture. This mixture was incubated for 60 minutes at 42 °C followed by 5 minutes incubation at 70 °C and then stored at -20 °C.

#### Quantification of cytokine mRNA expression.

Messenger RNA expression for IFN- $\gamma$ , TNF- $\alpha$  and IL-10 cytokines was quantified by relative real-time PCR assay. It was performed using SYBR Green I double-stranded DNA binding dye (Maxima SYBR Green/ROX qPCR Master Mix (2X) kit, Thermo-Scientific, USA) with 2  $\mu$ l of sample containing 80 ng cDNA, which was added to a 23  $\mu$ l reaction mixture including 0.35  $\mu$ l of 10 pmol of each primer, 12  $\mu$ l of SYBR Green I, 1.5  $\mu$ l of 50 mM MgCl<sub>2</sub> and 8.8  $\mu$ l of DDW. Then the mixture was run in a Light cycler (Applied Biosystems 7300, Real-time PCR System, USA) under the following conditions: an initial denaturation at 95 °C for 10 min, followed by 45 cycles of 30 s at 95 °C for DNA denaturation, 20 s at 58°C for annealing of primers, 30 s at 72 °C for primer extension. The melting protocol consisted of heating from 60 °C to 95 °C at a rate of 0.5 °C per step, and each step was held for 1 s for data acquisition, and finally cooling to 40 °C as the cooling program. The melting curves of all final real-time PCR products were analyzed for determination of genuine products and contamination by nonspecific products and primer dimers.

**Analysis of Real-time PCR data.** For each cytokine gene, it was confirmed that the efficiency of amplification was similar with  $\beta$ -actin gene (data not shown), permitting real-time PCR data were analysed using the comparative CT method ( $\Delta\Delta$ CT) (Schmittgen & Livak 2008). Briefly, first the difference ( $\Delta$ CT) between the CT values of the interest gene and the internal control gene ( $\beta$ -actin) are calculated for each sample to be quantified. The comparative  $\Delta\Delta$ CT calculation involves finding the difference between the treated sample  $\Delta$ CT and the control sample  $\Delta$ CT. The last step in quantification is to transform these values to absolute values. The formula for this is: Fold change =  $2^{-\Delta\Delta$ CT}, where:

$$\Delta\Delta$$
CT = [(CT<sub>gene of interest</sub> - CT <sub>$\beta$ -actin</sub>)<sub>vaccinated sample</sub> - (CT<sub>gene of interest</sub> - CT <sub>$\beta$ -actin</sub>)<sub>control sample</sub>].

**Statistical analysis.** The statistical significance of cytokine mRNA expression between blood samplings was tested by Student's t test. Differences between of them were considered significant if probability values of P<0.05 were obtained. The statistical tests were performed with SAS software (SAS Institute, USA, 2001).

## RESULTS

Blood samples taken from all guinea pigs post-vaccination were analyzed for mRNA expression of IFN- $\gamma$ , TNF- $\alpha$  and IL-10 genes. Findings of the real-time PCR indicated that all of these genes were upregulated. Cytokine mRNA expression of TNF- $\alpha$  and IL-10 significantly increased (P<0.05) in day 28th

**Table 1.** Real-time PCR primers used for the quantification of cytokines

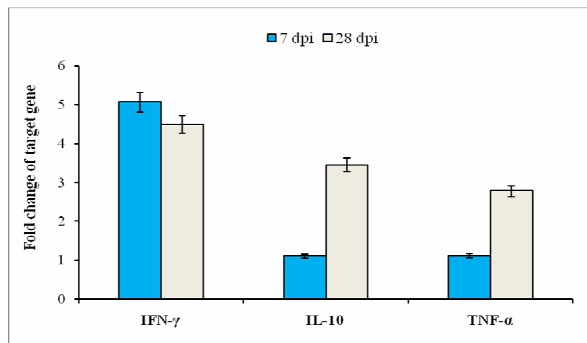
Target gene	Primer set	Primer length (bp)	Product length (bp)	Annealing temperature (°C)
IFN- $\gamma$	F-5' AAGACAACAGCAGCAACAAGGTG 3'	23	137	58
	R-5' TCGTTTCCTCTGGTTCGGTGAC 3'	22		
IL-10	F-5' GCACTACCAAAGCCACAAAGCAG 3'	23	133	58
	R-5' CTGTCCTCAGACTGGGTGTTCG 3'	22		
TNF- $\alpha$	F-5' CAGAAGACGCCATGAGCACAG 3'	21	134	58
	R-5' CACCAGCAGGAAGGAGAAGAGG 3'	22		
$\beta$ -actin	F-5' GCCTCACTCTCCACCTCCAG 3'	21	116	58
	R-5' AACGCAGCAAAGTAGTAACAGTCC 3'	24		

in comparison to the day 7th post the first vaccination.

**Table 2.** Mean  $\pm$  standard deviation of fold change for each cytokine in 7 and 28 days post the first injection (dpi).

Mean of fold change for interest gene $\pm$ SD			
Days	IFN- $\gamma$	IL-10	TNF- $\alpha$
7 dpi	5.071 $\pm$ 0.48 <sup>a</sup>	1.109 $\pm$ 0.10 <sup>b</sup>	1.125 $\pm$ 0.13 <sup>b</sup>
28 dpi	4.507 $\pm$ 0.44 <sup>b</sup>	3.461 $\pm$ 0.38 <sup>a</sup>	2.782 $\pm$ 0.28 <sup>a</sup>

Values with different superscripts in each column are significantly different ( $P < 0.05$ ).



**Figure 1.** Cytokine mRNA expression of guinea pigs were vaccinated to FMD inactivated vaccine. Blood samples were collected in 7 and 28 days post the first vaccination. Bars represent standard error of the mean. dpi: days post the first injection.

Nevertheless, IFN- $\gamma$  expression decreased in the second blood sampling compared to the first sampling ( $P < 0.05$ ). The bar graphs (Figure 1) that were designed to compare mRNA expression of each cytokine showed that IFN- $\gamma$  considerably increased ( $P < 0.05$ ) and IL-10 declined compared to the other cytokines. To sum up these finding, it seems that FMD type O inactivated vaccine has induced immune responses by increasing mRNA expression of IFN- $\gamma$ , TNF- $\alpha$  and IL-10 in vaccinated guinea pigs. Mean of fold change for each cytokine in 7 and 28 days post the first vaccination are presented in Table 2.

## DISCUSSION

Immune responses in vaccinated guinea pigs with FMD type O inactivated vaccine can be evaluated with the determination of cytokine mRNA expression

using real-time PCR. This study described the quantification of IFN- $\gamma$ , TNF- $\alpha$  and IL-10 expression in these animals. Although many effects of cytokines on cellular functions have been elucidated, little is known about the interactions between induced cytokines in response to the vaccine. Interleukin-10, which is a member of Th2 cytokines, can suppress Th1 functions by inhibiting cytokine production and downregulating the expression of them (Pestka et al., 2004). According to our results and previous investigations, it could be concluded that IL-10 might operate as negative modulators that while it was upregulated, the IFN- $\gamma$  which is a Th1 cell, downregulated. Decreased mRNA expression of IFN- $\gamma$  gene in day 28th post the first vaccination compared to previous time may be due to the fact that secretion of cytokines is usually transient. Furthermore, cytokines are not the pre-made molecules, but they product simultaneously with their genes transcription (Joseph et al., 2002). Similar to our results, upregulation of some cytokines in response to the vaccine or the virus of FMD has been confirmed in previous studies. Eble et al. (2006) and Barnard et al. (2005) demonstrated that both of IFN- $\gamma$  and IL-10 were induced by FMDV in pigs. Increased expression of IFN- $\gamma$  was also observed by Zhang et al. (2006) in cattle infected with FMDV but there was a slight increase in mRNA expression of TNF- $\alpha$ . When water buffalos were vaccinated with FMD inactivated vaccine, Mingala et al. (2009) reported that expression of IFN- $\gamma$ , IL-10 and TNF- $\alpha$  enhanced. Additionally, significantly increased cytokine mRNA expression was found by Zhang et al. (2009) in microdissected epithelia from the coronary band and tongue of FMDV-infected cattle. The factors such as presence of maternal antibody, nature and dose of antigen and the presence of adjuvant may affect the immune response to vaccination. Additionally, host age, nutritional factors, genetics and stress may be effective. The outcome of this study demonstrated that the inactivated vaccine induced immune responses by increasing mRNA expression of IFN- $\gamma$ , TNF- $\alpha$  and

IL-10 in guinea pig. It is expected that measurement the levels of these cytokine would provide valuable information about effects of DNA vaccines on immune system. This approach can lead to acceleration of the evaluation of DNA vaccines effects. This investigation would be an initial step towards using guinea pig model for examination of FMD DNA vaccines in future studies. It has certain advantages including the most sensitivity to FMDV and practical issues such as animal husbandry, containment space and cost. The identification of immune responses and vaccine efficacy are essential for the development of new vaccines or the optimization of immunization strategies using available vaccines. In the present study, real-time PCR was used as a powerful and suitable method for fast, accurate, sensitive and cost-effective cytokine mRNA expression analysis. Our findings showed significantly increased expression of cytokine mRNA in blood samples of vaccinated guinea pigs with FMD inactivated vaccine. Consequently, quantitation of these cytokines can be as a valuable adjunct for assessment effects of DNA vaccines effects on immune system.

### Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

### Conflict of Interest

The authors declare that they have no conflict of interest.

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