

## Evaluation of Cell-mediated Immune Response in PBMCs of Calves Vaccinated by Capri Pox Vaccines Using ELISA and Real-time RT-PCR

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### Abstract

**Background:** The analysis of antigen-specific cytokine expression has been considered to evaluate the immune responses and vaccines efficacy in recent years. The aim of this study was to compare the cell-mediated immune response characteristics of two Capri pox virus (CaPV) vaccines against lumpy skin disease in cattle.

**Materials and Methods:** Two Capri pox virus vaccines were administered to dairy cows of two farms and followed up to 5 weeks post vaccination. These vaccines were live attenuated Goat pox virus (GTP) Gorgan strain (n=20) and Sheep pox virus (SPP) Romanian strain (n=20). Cell-mediated immune response of vaccinated calves was evaluated using in vitro lymphocyte proliferation and IFN- $\gamma$  and IL-4 release assay after stimulation with recall vaccine strains, and in vivo cytokine expression in PBMCs by real-time PCR.

**Results:** Lymphocyte proliferation in GTP- and SPP-vaccinated groups began to increase till reached to its peak at third week post vaccination and then decreased in the weeks thereafter. Stimulation index in stimulated PBMCs in GTP-vaccinated calves was higher than SPP-vaccinated calves in all weeks, which indicated higher levels of immunogenicity produced by the GTP-vaccine in cattle.

Also, in both vaccinated groups the peak release of IFN- $\gamma$  and IL-4 proteins in cultured PBMCs in response to recall antigen was detected at week 3 post vaccination. Although the mean of the cytokine release in GTP-vaccinated calves was higher than SPP-vaccinated calves in all weeks of experiment, a significant difference was only observed at week 3 post vaccination ( $P<0.05$ ). In contrast, the IFN- $\gamma$  mRNA expression in PBMCs of vaccinated groups was induced early, peaked at week 1 post vaccination and decreased in the weeks thereafter, and this rate was higher in GTP-vaccinated calves compared with SPP-vaccinated calves in all weeks, but the significant difference was only found at week 3 post vaccination ( $P<0.05$ ). However, the IL-4 mRNA expression showed delayed induction and peaked at week 3, and unlike the SPP group, it remained at this level in GTP group, until the end of experiment. Also this rate of expression in GTP-vaccinated calves was higher than SPP-vaccinated calves in all weeks and had a significant increase at week 5 post vaccination ( $P<0.05$ ).

**Conclusion:** The findings show that due to induction of high level cell-mediated immune response in live attenuated GTP vaccine compared to SPP vaccine, GTP vaccine has a good immunogenic response, and therefore can be a better choice for vaccination against lumpy skin disease.

**Keywords:** Capri pox Virus, Cellular immunity, Real-time PCR, ELISA

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## Introduction

Capri pox viruses (CaPV) genus is composed of lumpy skin disease (LSD) virus, sheep pox (SPP) virus, and goat pox (GTP) virus, which are economically important viral diseases of domestic ruminants, primarily affecting cattle, sheep and goats, respectively (1). According to many studies, it has been proven that CaPV strains have a high genetic similarity and share a major neutralizing site, so that the animals infected with one strain of CaPV family and survived from it, will become resistant to infection with any other strains. Therefore, the use of vaccine strains of CaPV derived from sheep and goat would be useful to protect cattle against LSD (2-4). Since, the suitable way to prevent and control CaPV diseases especially lumpy skin disease is vaccination, recently developed live attenuated CaPV vaccine strains are used for the prevention and control of LSD. These vaccines provide good protection depending on stimulating cellular and humoral immunity.

Functional analysis of the effector cells generated upon activation of peripheral blood mononuclear cells (PBMCs) with vaccine strains should contribute to the elucidation of the basic mechanism responsible for the stimulation of the immune system (5, 6). For this purpose, cytokine production analyses focused on the secretion of Th1-like cytokine: IFN- $\gamma$  and Th2-like cytokine: IL-4, because they play an important role in humoral and cellular immune response against infectious agents.

Hence, in the present study, the cell-mediated immune response of two live attenuated CaPV vaccines, and how these heterologous vaccines stimulated the immune response against LSD virus were evaluated. Accordingly, after the isolation of PBMCs of vaccinated calves in weeks post vaccination, in the first step, we measured in vivo IFN- $\gamma$  and IL-4 mRNA expression; and in the second step, the PBMCs of vaccinated calves were re-stimulated in vitro by vaccine strains to identify lymphocyte proliferation and IFN- $\gamma$  and IL-4 cytokine release, in order to determine the relationship between in vivo and in vitro cytokine levels.

## Materials and methods

### Animal vaccination

In this study animals consisted of Holstein breed male calves with approximately 4-6 months of age without any known previous exposure to LSD. Calves were selected from two dairy farms, and were divided into two groups: treated group in farm 1 (n=20) received GTP vaccine, and treated group in farm 2 (n=20) received SPP vaccine. The control

groups (n=4) in each farm received PBS (phosphate buffered saline) alone. These vaccines were live attenuated Romanian-sheep pox virus and Gorgan-goat pox virus, which were produced by the Razi vaccine and serum research institute (RVSRI) of Iran, and one dose of each vaccine for goat and sheep contained  $10^{5.2}$  TCID<sub>50</sub>/ml of virus. Each lyophilized vaccine based on ten-fold dose of virus was reconstituted in distilled water according to the manufacturer's instructions (7), and treated groups received the prepared vaccine through subcutaneous according to the manufacturer's instructions.

### Virus preparation

All vaccine strains were obtained from RVSRI. The cultivation of sheep and goat pox viruses was carried out according to the standard protocol of the department of animal viral vaccines of RVSRI following OIE (Office International des Epizooties) manual (7, 8), and the titer of prepared virus stock was calculated by Reed-Muench method (9). For purification and inactivation of viruses, after the removal of cell debris, the harvested virus was concentrated by ultracentrifugation in sucrose density gradient, and after the titration, the virus inactivation was carried out according to OIE manual (8, 10).

### PBMCs isolation and Lymphocyte proliferation assay

10-15 ml of whole blood samples were taken weekly from the jugular vein of calves and the PBMCs were isolated within 4-8 h after bleeding by density gradient centrifugation according to standard protocol (11, 12). Viable cells percent was determined by staining with trypan blue and adjusted to concentration of  $2 \times 10^6$  cells/ml in RPMI complete medium (13, 14). Lymphocyte proliferation assays were done in quadruplicate in 96-well flat-bottom plates using MTT assay as previously described (11). Briefly, PBMCs of each vaccinated calve at a concentration of  $2 \times 10^5$  cells/well were added to each well and stimulated with inactivated vaccine strain viruses at a MOI (Multiplicity of Infection) of 0.1 depending on the optimal stimulating capacity of virus (data not shown). The PBMCs were cultured at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> for 4 days. After the incubation time, lymphocyte proliferation assay was carried out according to the instructions Kit (cell proliferation kit, Roche, Germany). The amount of MTT formazan produced during the incubation was measured by an ELISA reader (Bio-Tek ELx800) at a test wavelength of 550 nm and a reference wavelength of 630 nm. The results were calculated on the optical density and expressed as a stimulation index (SI) (11, 15). The SI was calculated as follows:

SI = (mean OD550 of stimulated PBMCs) - (mean

OD550 of blank)/ (mean OD550 of unstimulated PBMCs)

*Cytokine (mRNA) gene extraction*

PBMCs of vaccinated calves were used weekly to cellular mRNA extraction using Oligotex Direct mRNA Mini kit (Qiagen), as recommended by the manufacturer’s instructions of the Kit. The purity and quantity of mRNA was assessed using Nano Drop ND-1000 spectrophotometer (Thermo Scientific, MA, USA), so that A280:A260 ratio in range of 1.8-2.0 and A260:A230 ratio in range of 2.0-2.2 were considered pure and used for cDNA synthesis (16, 17). Synthesis of first-strand cDNA was carried out on Veriti thermal cycler (Applied Biosystems, CA, USA) by using random hexamer primers and Super

Script III First-strand cDNA synthesis kit (Invitrogen, CA, USA) according to the manufacturer’s instructions of the Kit.

*Real-time PCR assay*

The Real-time RT-PCR was catalyzed using Fast-Start Essential DNA SYBR Green Master kit (Roche Diagnostics, Mannheim, Germany), with Light cycler 96 (Roche Diagnostics, Mannheim, Germany) as recommended by the manufacturer’s instructions. The primers of IL-4 and IFN- $\gamma$  genes and GAPDH gene as an internal control were designed from NCBI Gen Bank database (Table 1).

**Table 1.** Sequences of oligonucleotide primers and optimal annealing temperature for real-time RT-PCR.

Gene	Primer sequence <sup>a</sup> (forward and reverse)	Product size (bp)	Anneal tem. °C	Accession number
IL-4	F: 5'- CAA AGA ACA CAA CTG AGA AG -3' R: 5'- AGG TCT TTC AGC GTA CTT GT -3'	181	54	M77120
IFN- $\gamma$	F:5'- ATA ACC AGG TCA TTC AAA GG -3' R: 5'- ATT CTG ACT TCT CTT CCG CT -3'	218	52	M29867
GAPDH	F:5'-GGC GTG AAC CAC GAG AAG TAT AA -3' R: 5'- CCC TCC ACG ATG CCA AAG T -3'	194	56	U22385

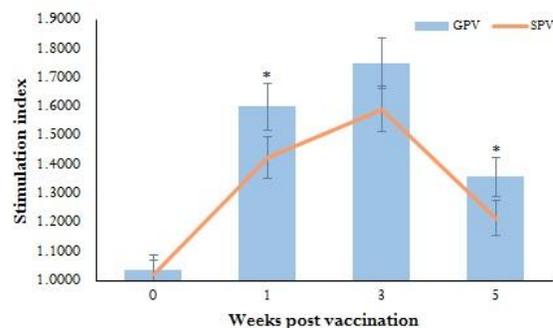
The amplification reactions were carried out in a final volume of 20  $\mu$ l as previously described (16, 17). The thermal profile used for the reactions of cytokines was to heat samples for 1 min at 95 °C as the initial denaturation program; followed by 50 concurrent cycles involving 94 °C for 1 sec as denaturation program; 54 °C for 5 sec for IL-4 and 52 °C for 5 sec for IFN- $\gamma$  and 56 °C for 20 sec for GAPDH as annealing program; 72 °C for 10 sec as extension program; and finally cooling to 40 °C as the cooling program. Gene expression was determined as fold increase given by the  $2^{-\Delta\Delta CT}$  formula (16-18).

*ELISA assay*

IL-4 and IFN- $\gamma$  cytokines concentrations in supernatants of stimulated PBMCs were measured for each vaccinated group. Cell-free supernatants were collected on the fourth day of culture (11, 13) and concentrations of IFN- $\gamma$  and IL-4 cytokines were measured using commercially available ELISA-kits (USCN Life Science Inc. China). Assays were performed according to the manufacturer’s protocol and with reference to standards provided by the manufacturers. The limits of detection (LOD) for the individual assays were as follow: IL-4, 6.2pg/ml and IFN- $\gamma$ , 12.8pg/ml.

**Statistical analysis**

In this study, data were analyzed by the analysis of variance (one-way ANOVA) using general linear model procedures. A P-value of less than 0.05 was considered significant.



**Figure 1.** Lymphocyte proliferation index of vaccinated calves-PBMCs in response to inactivated GTP and SPP-virus at 0 (before vaccination), 1, 3 and 5 weeks post vaccination.

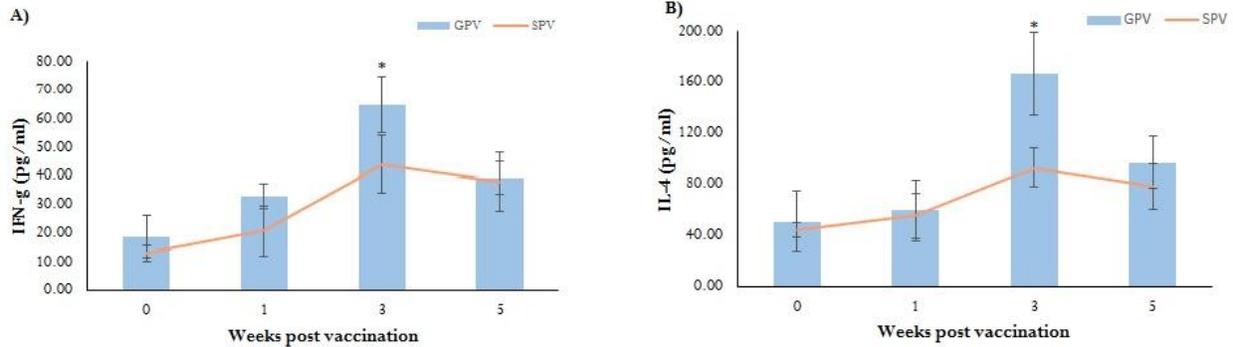
**Results**

*Lymphocyte proliferation and cytokine production of stimulated-PBMCs*

The PBMCs from vaccinated calves receiving whole virus vaccine strains will respond to inactivated virus

in an in vitro re-stimulation assay (19). The PBMCs of vaccinated calves did show higher proliferation than control group (non-vaccinated calves) (data not shown) in all time points of the experiment. The high

lymphocyte proliferative rate in response to PHA represented the positive control and indicated good viability of the cultured PBMCs.



**Figure 2.** IFN-  $\gamma$  (A) and IL-4 (B) concentration in the supernatants of stimulated-PBMCs with inactivated GTP and SPP-virus at 0 (before vaccination), 1, 3 and 5 weeks post vaccination. Cytokines concentration was measured using ELISA kit.

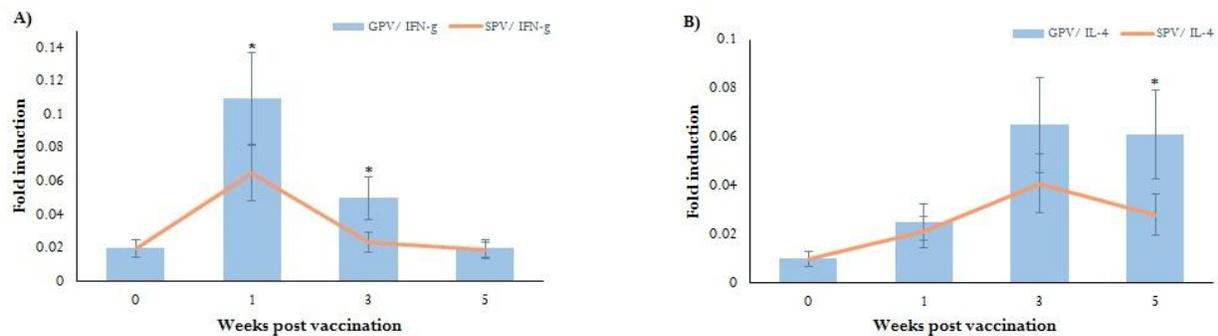
According to the results presented in Figure 1, the lymphocyte proliferation level of vaccinated calves in response to vaccine strains reached its highest level in the third week post vaccination. Although proliferative level in GTP-vaccinated calves was higher than SPP-vaccinated calves in all weeks of the experiment, this difference was only significant at weeks 1 and 5 post vaccination ( $P < 0.05$ ).

demonstrated a wide range of values. The IFN- $\gamma$  and IL-4 production in each vaccinated group increased at week 1 post vaccination, peaked at week 3 and decreased in the period thereafter, while in control group there was no increase in any time-points. Although the mean of the IFN- $\gamma$  and IL-4 production level in GTP-vaccinated calves was higher than SPP-vaccinated calves in all weeks of the experiment, although a significant difference was only observed at week 3 post vaccination ( $p < 0.05$ ).

Lymphocyte activation was further studied by analyzing cytokine production using ELISA assays in PBMCs stimulated with inactivated virus. The Th1-like cytokine (IFN- $\gamma$ ) and the Th2-like cytokine (IL-4) were found in variable levels in the supernatant of virus-stimulated PBMCs in both GTP-and SPP-vaccinated groups. The production of IL-4 and IFN- $\gamma$  cytokines in virus-stimulated PBMCs significantly increased compared with the unstimulated cultures (control group) at all-time points (data not shown). The mean values of IFN- $\gamma$  and IL-4 production of each vaccinated group are shown in Figure 2A-B, and

*Quantitative PCR (QPCR) of cytokine gene*

The PBMCs of vaccinated calves were isolated from the whole blood at different weeks post vaccination. Although the expression of cytokines in the whole blood was relatively low, this rate increased after vaccination in comparison to the control group. IFN- $\gamma$  mRNA gene expression in both vaccinated groups was started from the first days after inoculation and peaked at week 1 post vaccination, and then gradually decreased until the end of the study.



**Figure 3.** The mRNA gene expression levels of IFN- $\gamma$  and IL-4 in PBMCs isolated from whole blood in the weeks post vaccination. Cytokines expression was measured using Real-time PCR. The bars (+SEM) shows the fold increase of gene expression in vaccinated groups.

Also, a significant difference of IFN- $\gamma$  gene expression between the vaccinated groups was detected at weeks 1 and 3 post vaccination and this difference in GTP-vaccinated calves was higher than SPP-vaccinated calves ( $p < 0.05$ ) (Figure 3A). In contrast, IL-4 mRNA gene expression in both vaccinated groups was started from the first week post vaccination and peaked at week 3 post vaccination. A significant difference between the groups was detected at week 5 post vaccination ( $p < 0.05$ ), and this difference in GTP-vaccinated calves was higher than SPP-vaccinated calves ( $p < 0.05$ ) (Figure 3B).

### Discussion

The aim of this study was to compare the cell-mediated immune responses of two Capri pox virus-vaccine strains against LSD virus in dairy cattle population. In this research we sought to provide detail on the characteristics of the GTP and SPP vaccine-induced cellular immune response by measurement of target cytokines, because they are critical parameters in immune response and can be related to the durability of protection.

There is evidence that cellular immunity plays an important role against Capri pox viruses besides humeral immunity. Hence, cell-mediated responses of vaccinated calves were demonstrated after vaccination using lymphocyte MTT proliferation assay in which responses are probably mainly attributable to T-helper cells (11, 20). Lymphocyte proliferation of cultured PBMCs were analyzed after re-stimulation with inactivated vaccine strains. Stimulation index of PBMCs in GTP-vaccinated calves was higher than in SPP-vaccinated calves in all time points of experiment. These results are consistent with the findings in other papers (21, 22).

In many previous studies, immune responses of Capri pox viruses have been investigated, but the functional role of induced cytokines by vaccination and how they contribute to protective responses have not been clearly identified. Since cytokines are generally produced locally and at low levels, it might be difficult to detect them systemically; hence, in addition to the cytokine genes expression in whole blood, their production rate was also evaluated in vitro in cultured PBMCs with virus. By analyzing these results, we can achieve the actual structure of gene expression and production of cytokines in response to the vaccine antigens. Because in many studies it has been proven that the cytokine genes expression is not always directly related to their production, and the transcribed genes expression may be untranslated or suppressed by various intracellular factors.

In assessing the cytokine genes expression in whole blood-derived PBMCs in both vaccinated groups, a significant increase in IFN- $\gamma$  expression was found at week 1 post-vaccination. Also, a significant difference between the groups was detected at weeks 1 and 3, and this difference in GTP-vaccinated calves was higher than SPP-vaccinated calves ( $p < 0.05$ ). These results indicate the onset of cellular stimuli in the early days after vaccination and the expression of inflammatory cytokines, especially IFN- $\gamma$ . These results are consistent with the results observed in the early days after inoculation of the virus and the onset of clinical symptoms such as fever, inflammation and swelling in the injection site. In contrast, for IL-4 expression a significant increase was shown at week 3 post-vaccination. Also, a significant difference between the vaccinated groups was detected at week 5, and this difference in GTP-vaccinated calves was higher than SPP-vaccinated calves ( $p < 0.05$ ).

In assessing the cytokine production after re-stimulation of PBMCs with vaccines strains, the production of IL-4 and IFN- $\gamma$  cytokines were observed at week 1, peaked at week 3 and decreased in the days thereafter, and a significant difference between the groups was detected at week 3 post vaccination ( $p < 0.05$ ). From the above result, it can be deduced that GTP vaccine give good immunogenicity, inducing a higher level of IL-4 and IFN- $\gamma$  in response to vaccine strain compared with SPP vaccine. In other hand, correlation of Th1 and Th2 cytokines induced by the GTP vaccine was higher than SPP vaccine.

### Conclusion

Based on the above study, we concluded that both GTP and SPP vaccines induced cell-mediated immune response, with the induction of both a Th1-like and a Th2-like activity; however, GTP vaccine was considered a more suitable vaccine to control the LSD disease in the field due to inducing high level of IFN- $\gamma$  and IL-4 expression and production level, and also higher lymphocyte proliferation.

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### Author Contributions

All authors of this article have the same contribution to perform this project.

### Conflict of interest

The authors declare no conflict of interest.

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