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Quantitation of cytokine gene expression by real time PCR in bovine milk and colostrum cells from cows immunized with a bovine rotavirus VP6 experimental vaccine

Q1 D.D. Gonzalez*, A. Rimondi, M.S. Perez Aguirreburualde, M. Mozgovoj, D. Bellido, A. Wigdorovitz, M.J. Dus Santos

Q2 *Instituto de Virología-CICVyA, INTA Castelar, Las cabañas y De Los Reseros S/N, CP1712 Buenos Aires, Argentina*

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ABSTRACT

In a previous work, VP6 recombinant protein was produced using baculovirus system and it was evaluated in a colostrum-deprived calf model. This vaccine was able to protect calves against viral challenge without inducing neutralizing antibodies (NAb), suggesting that another immunological effectors were involved in the protection observed. In this work, groups of cows ($n = 4$) were immunized in the last third of gestation with a bovine rotavirus (BRV) experimental vaccine and with a VP6 subunit vaccine. At birth, colostrums from vaccinated and non-vaccinated cows were processed and viable colostral mononuclear cells were obtained. With the purpose of determining the cytokine patterns generated by cells from immune secretions (colostrums and milk), a relative quantification by real time PCR was standardized. Quantitative real time PCR (qPCR) was used to determine transcript levels of IL-4, IL-6, IL-10, IL-12, IFN-γ and IFN-α from these cells. Colostral and milk mononuclear cells expressed a different cytokine transcript expression pattern regarding the vaccine used. These results demonstrated that the colostral cellular population was active and could exert its action influencing the final immune response.

1. Introduction

Bovine colostral secretions contain nutrients, minerals, trace elements, (pre-)vitamins, immunoglobulins, cytokines and cells (1×10^6–3×10^6 cells/ml, almost exclusively leucocytes), which are completely functional after absorption by the calf (Riedel-Caspari, 1993). Due to the early susceptibility to rotavirus (RV) infection, prevention strategies in calves are based on increasing the levels of passive immunity. This can be achieved by vaccination of pregnant cows during the last third of gestation (Cornaglia et al., 1992). Therefore, protection against bovine rotavirus (BRV) infection is only related to the passive immunity levels acquired by consumption of colostrum and milk.

In a recent study from our group (Gonzalez et al., 2010) we have demonstrated that a recombinant subunit vaccine, which contains the VP6 protein expressed in the baculovirus system, induced a passive protective immune response which was transferred by colostrum to the calf. Similar results were obtained by many authors in the murine model (Choi et al., 1999, 2002a, 2002b; Dong et al., 2005; McNeal et al., 2007). Since VP6 protein does not induce neutralizing antibodies (NAb), it has been hypothesized that other mechanisms could contribute for protection against RV infection and disease. McNeal and others (McNeal et al., 2002) have performed vaccination studies in knockout mice for B cells, and they have demonstrated that the immunogenicity conferred with a VP6 subunit vaccine could be removed when mice were depleted of CD4 T cells. Given that CD4 T cells collaborate in the immune response stimulation through the cytokines network, these molecules could constitute potential effectors of immunity against RV infection.

Cytokines messenger RNA (mRNA) are expressed in low quantities, therefore, accurate methods are required for their measurement. Several methods allows quantitation of cytokine expression at the protein level (ELISA, Elispot, biological assays, intracellular cytokine staining) (Kabilan et al., 1990) and at the mRNA level (Northern blots, *in situ* hybridization, ribonuclease protection assay, reverse transcriptase polymerase chain reaction (RT-PCR)) (Dallman et al., 1991) of peripheral blood mononuclear cells (PBMC), peripheral blood lymphocyte subpopulations and in tissues. Northern blots and ribonuclease protection assays require large amounts of RNA that may constitute a limiting factor when small amounts of tissue samples or blood cell preparations are analyzed. PCR overcomes these limitations and the fact that several nucleic acid molecules can be amplified up to microgram amounts opens the possibility to study gene regulation even in a single cell.

* Corresponding author. Tel./fax: +54 11 4621 8773x156.
E-mail address: dgonzalez@cnia.inta.gov.ar (D.D. Gonzalez).

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In that sense, quantitative real time PCR (qPCR) is a high sensitivity technique which results useful to quantify minimal physiologic changes in genes expression. It was reported that qPCR has a limit of detection from 10 to 100 folds higher than other methods (Bustin, 2000), and nowadays it is the technique used in most veterinary studies due to the good reproducibility and wide range of quantification (Pfaffl et al., 2003).

Until now, there are few reports about the presence of pathogen-specific cells and their cytokines gene expression in bovine immune colostrums, and none of them involve immunization with BRV vaccines. In this work, we explored the cytokine gene expression pattern in colostral cells from cows immunized with a VP6 subunit vaccine in comparison with cows immunized with a conventional BRV vaccine. For this purpose a qPCR technique was standardized to quantify the cytokine mRNA expression using a relative method. This technique was able to analyze, with high sensitivity, the cytokine components in colostrum after vaccination.

2. Materials and methods

2.1. Immunization and samples collection

Twelve Holando-Argentine cows from 2nd milking, and with basal Ab titers against BRV (Parreno et al., 2004) were used in this study. Cows were housing in a controlled facility so all of them were evaluated by SCC (somatic cell count).

The vaccines were formulated using either BRV or recombinant VP6 as described previously (Gonzalez et al., 2010). Four cows were vaccinated intrammuscullarly (IM) with the VP6 subunit vaccine and another four cows were immunized with the BRV vaccine by the same route. Both groups received three doses of 5 ml, applicated in the last third of gestation (at 45, 30, and 15 days before calving). Four cows remained unvaccinated and were included as control calibrator group.

Sera from cows were collected at 45 and 15 days pre-partum, and at calving. Antibodies in serum against BRV were evaluated by ELISA (Parreno et al., 2004) and virus neutralizing test (To et al., 1998).

First milking colostrum was collected from each dam at calving. Milk samples were taken on day 5 post-calving (+5).

2.2. Isolation of mononuclear cells

2.2.1. Colostrum and milk

Aliquots of 25 ml from both secretions (colostrum and milk) were mixed with 25 ml of PBS pH = 7.2 and then were centrifuged at 500g for 20 min at 4 °C. Following that, two different procedures were carried out:

Procedure A: The pellet was resuspended with 13.5 ml of isotonic Percoll (GE Healthcare) plus 36.5 ml of PBS 1× and centrifuged at 1620g for 30 min at 4 °C. Then, the pellet was resuspended in Percoll 43% (7 ml). This suspension was seeded on a Percoll 70% solution and centrifuged (without brake) at 2205g for 20 min at 4 °C. The cells were obtained at the interface between two layers and then they were washed with RPMI 1640 medium (SIGMA).

Procedure B: The cellular pellet was resuspended in PBS pH = 7.2 and these cells were centrifuged in a Ficoll-Paque PLUS (GE Healthcare) density gradient according to the manufacture's instructions.

2.3. RNA extraction and DNase I treatment

Total ribonucleic acid (RNA) extraction was performed from the mononuclear cells obtained, using RNeasy Mini kit spin columns according to the manufacturer's instructions (Qiagen). RNA was eluted in 50 μl of RNase-free water. To remove any possible contaminating genomic DNA (gDNA), the RNA sample was treated with RNase-Free DNase I (Promega) according to the manufacturer's instructions. The concentration and purity of nucleic acid were determinated with the OD value at 260 nm and the A260/A280 coefficient respectively in a NanoDrop 1000 Spectrophotometer (Thermo Scientific). Only RNA of high purity (A260/A280 in the range of 1.8–2.0 values) was used. The extracted RNAs were kept at −80 °C until use. The integrity of the RNA were determinated by agarose gel electrophoresis (data not shown).

2.4. Reverse transcription (RT)

The reverse transcription (RT) reaction mix was prepared by adding 0.1 μg total RNA, 400 ng Oligo $(dT)_{15}$ (Promega) and 1 μl of a mix with 10 mM of each dNTP. The final volume was adjusted to 13 μl with RNase-free water and heated at 65 °C for 5 min. After cooled on ice for 1 min, 200 U SuperScript III RT Reverse (Invitrogen), 1 μl of 0.1 M DTT and 40 U of RNAsin (Promega) were added. The final volume was adjusted to 20 μl with RNAse free water. The reaction mix was subjected to 50 °C for 60 min and then the temperature was raised to 70 °C for 15 min to inactivate the reverse transcriptase. The copy DNA (cDNA) was used immediately or stored at −20 °C until use.

2.5. qPCR

To quantify the cytokines mRNA expression in the samples, a real time TaqMan PCR relative method was used. A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was simultaneously processed in Real-time TaqMan PCR for each sample as an endogenous control and in accordance to others authors, who worked with colostrum and milk cells (Alluwaimi et al., 2003; Gramsci, 2007; Leutenegger et al., 2000; Peli et al., 2004).

2.6. Primer design

Primers for seven specific bovine genes were designed to have an equal annealing temperature of 60 °C (Table 1). Table 1 lists the corresponding accession number of the 7 genes, with their respective forward, reverse and probe primer.

2.7. Real time PCR

The PCR reactions mix was prepared containing 500 nM of each primer, 100 nM of the TaqMan probe, 10 ng of the diluted cDNA sample and commercially available PCR Mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems), in a final volume of 25 μl. The samples were placed in 96-well plates and amplified in an Applied Byosistems RT PCR 7500 equipment. Amplification was performed under the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C.

2.8. Determination of amplification efficiency

A standard curve method was employed to determine the amplification efficiencies of GAPDH and cytokines cDNAs. Briefly, five serials dilutions of cDNA were amplified in triplicate. The resulting CT values were plotted against the dilution of total RNA and the regression line was calculated. The slopes of the regression line for each cytokine gene were used to determine the amplification efficiency (Pfaffl, 2001): Efficiency (E) = 10 (−1/slope) (Eq. (1)).

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Table 1
Primers sequences (forward and reverse) and probe for quantitative real time PCR (qPCR) technique.

	Primer forward	Primer reverse	Probe
GAPDH	GCATCGTGGAGGGACTTATGA	GGGCCATCCACAGTCTTCTG	CACTGTCCACGCCATCACTGCCA
IL-4	GCTGCCTGTAGCAGACGTCTT	AATTCCAACCCTGCAGAAGGT	TGCCCCAAAGAACACAACTGAGAAGGA
IL-6	GGGCTCCCATGATTGTGGTA	GTGTGCCCAGTGGACAGGTT	TTCCTGGGCATTCCCTCCTCTGGT
IL-10	CTTGTCGGAAATGATCCAGTTTT	TTCACGTGCTCCTTGATGTCA	CCACAGGCTGAGAACCACGGGC
Il-12	CCAAAGTCACATGCCACAAGG	CTGTAGTAGCGGTCCCGGG	TGCCAACGTCCGCGTGCAA
IFN-α	GTGAGGAAATACTTCCACAGACTCACT	TGARGAAGAGAAGGCTCTCATGA	TGCTCTGACAACCTCCCAGGCACA
IFN-γ	CAGAAAGCGGAAGAGAAGTCAGA	TGCAGGCAGGAGGACCAT	CTCTTTCGAGGCCGGAGAGCATCAAC

The accession number *(AN)* in the database (NCBI) corresponding to: (GAPDH, AN = NM_001034034.2); (IL-4, AN = EU276069.1); (IL-6,AN = BC123577.1); (IL-10,AN = EU276074.1); (IL-12,AN = EU276076.1); (IFNα, AN = EU276064.1); (IFNγ,AN = EU277737.1).

2.9. Relative quantification

Gene expression was measured by relative quantification, which compares the threshold cycle (Ct) of the sample of interest (from vaccinated animals) with the Ct generated by a reference sample referred to as the calibrator (from non-vaccinated animals). The expression of each gene was analyzed using the relative quantification method described by Pfaffl (Pfaffl, 2001). In brief, a slope was determined from the exponential phase, under the optimized real-time PCR amplification condition, of each target cytokine gene or the reference gene (GAPDH).

The expression of selected cytokine genes was normalized to that of GAPDH, at each time point and converted to the relative expression ratio (fold of induction), according to the following formulae:

$$\text{Fold of induction(R value)} = (E_{target})^{\Delta Cttarget(calibrator-sample)} / (E_{ref})^{\Delta Ctreference(calibrator-sample)} \quad (1)$$

where: $E_{target:}$:PCR efficiency of the target.; ΔCt target (calibrator-sample) = (Ct_{target}) in the calibrator- (Ct_{target}) in the sample; E_{ref}:PCR efficiency of the reference.; ΔCt reference (calibrator-sample) = ($Ct_{reference}$) in the calibrator- ($Ct_{reference}$) in the sample.

3. Results

3.1. Animals immunized

Somatic cell count, or a parameter derived from this count, is often used to distinguish udder health (80% in uninfected quarters, 99% in mastitic quarters) and milk quality (Pillai et al., 2001; Sordillo et al., 1997). Somatic cells (lymphocytes, macrophages, polymorphonuclear cells and some epithelial cells) are therefore a reflection of the inflammatory response to an intramammary infection or another trigger of the immune system. The animals in our experience were housed in a controlled facility that routinely evaluates this parameter in their pregnant cows. The animals' quarters were healthy and no presence of mastitis was detected (data not shown).

Both vaccinated groups showed seroconversion of IgG antibodies to BRV in serum. Specifically, in cows immunized with the VP6 subunit vaccine, IgG1 increased at 15 days before calving and then declined by day 0. On the other hand, IgG2 titers at 15 days prepartum were higher than at the beginning of the experience (day 45 before calving). Cows vaccinated with the conventional BRV vaccine increased the NAb level, while animals immunized with the VP6 vaccine do not change their NAb titers in this period (Fig. 1) (Gonzalez et al., 2010).

3.2. Isolation of mononuclear cells

The purification procedure of mononuclear cells was standardized with the goal of obtaining the highest quantity of viable cells. After processing the colostrums two procedures were tested. The highest purification efficacy (17-fold higher) was obtained with the Percoll procedure in comparison with Ficoll-Paque PLUS extraction methodology. Purification of mononuclear cells from milk resulted 3 times more efficient with the Percoll procedure than with Ficoll-Paque PLUS one (Table 2). The yield obtained from colostrum and milk was in concordance with the bibliography (Nagaeva et al., 2002; Pertoft, 2000; Schmaltz et al., 1996) but there were considerably differences between the two procedures. When we used the Percoll procedure, we worked with two different gradients (43% and 70%); this probably allowed obtaining a higher purification yield in cells that were in a fatty environment and moreover, preserved the cellular viability. Cells from colostrum and milk reached an 85% of viability (as determinated with Trypan Blue exclusion stain).

3.3. Standardization of qPCR

3.3.1. qPCR efficiency

Plots between initials concentrations of cDNA (log value) versus Ct values, for each cytokine, gave a slope and an R^2 value, which were utilized for determining an E value for efficiency calculations. This parameter specified if the qPCR for each cytokine was acceptable or not. An E value close to 100% is the best indicator for a robust and reproducible assay. However, values between 90%–110% are also allowed (Liu and Saint, 2002; Meijerink et al., 2001).

For amounts of cDNA ranging from 5 pg to 10 ng, the transcritps showed a high efficiency. The R^2 value indicated the variability between intra-assay replicates; R^2 must be higher than 0.980 for an accurate assay. When higher quantities of cDNA were utilized, R^2 gave values < 0.980 (Table 3).

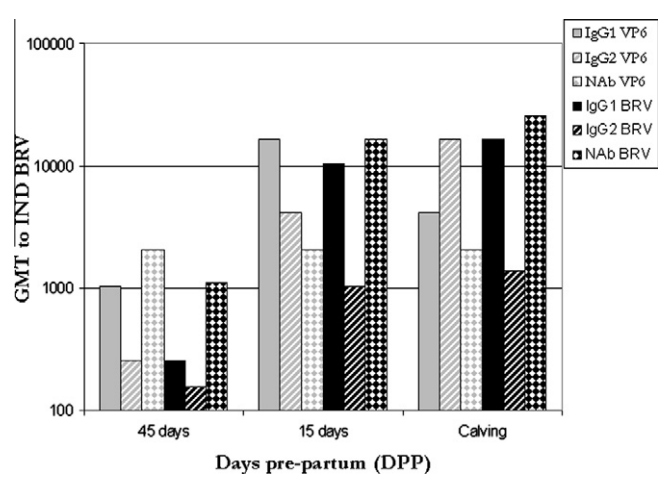

Fig. 1. Ab titers in cows vaccinated with BRV and VP6 vaccines. Geometric mean antibody titer (GMT) to IND BRV detected at different days pre-partum in serum of cows immunized with the subunit vaccine (tones of grey) and the BRV vaccine (tones of black).

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Table 2
Purification of mononuclear cells from milk and colostrum by different treatments for milk and colostrum.

	COLOSTRUM		MILK	
Extraction Methodology	Ficoll–Paque PLUS	Percoll 43%/70%	Ficoll–Paque PLUS	Percoll 43%/70%
Sample Volume (mean volume)	3 l	1 l	2 l	1.5 l
Cells (mean cells)	3 exp 6	1.7 exp 7	1.4 exp 7	3.6 exp 7
(Mean cells)/l of secretion	1 exp 6	1.7 exp 7	7 exp 6	2.4 exp 7
Extraction Ratio (Percoll/Ficoll–Paque)	**17-fold**		**3-fold**	
Cell viability	85%		85%	

Ct values obtained were from 20 to 35. Those values were expected for a correct determination of genes expression. For this reason we have set a final concentration of 5 ng/μl of cDNA per reaction and a final concentration of 100 and 500 nM for probe and primers, respectively.

3.4. Determination of R value (Pfaffl equation)

For a relative quantitation by the comparative CT method, values are expressed relative to a calibrator (non-vaccinated animals). The CT for the target gene and the CT for the internal control were determined for both the vaccinated groups and the calibrator. The expression of selected cytokine genes was normalized to the reference gene, bovine GAPDH. This was done to normalize the process for differences in extraction efficiency, for mRNA degradation in the starting material, and for reverse transcriptase reaction efficacy. Finally, quantification was done using the Pfaffl equation (Eq. (2)), which incorporates the qPCR efficiencies (Eq. (1)), for a dimensionless determination of R value (the n-fold difference relative to calibrator cDNA). The *R* values obtained from mononuclear cells in each experimental group (VP6 and BRV), at specific times and from different samples (colostrum and milk), are described in Table 4.

When the presence of IL-6 transcripts in colostral mononuclear cells from each group was compared, a *R* value roughly 5-fold higher could be observed in animals vaccinated with BRV (R_{BRV} col. = 2.55) with respect to the R value of animals vaccinated with VP6 (R_{VP6} col. = 0.55). That ratio between groups was maintained when the analysis was performed in milk (day + 5) (R_{BRV} milk = 16.58; R_{VP6} milk = 3.9).

IL-12 promotes the IFN-γ production, the polarization of CD4 + T cells bias Th1 and the development of cytotoxic CD8 + T cells. In this work, colostrum cells from cows immunized with the BRV vaccine showed an *R* value higher for IL-12 (R_{BRV} col. = 0.84) in comparison with the cows immunized with the VP6 vaccine (R_{VP6} col. = 0.27). Unfortunately, it was not possible to determinate the *R* value for IL-12 in milk (ND at both cases).

The presence of IFN-γ transcripts could be also observed in colostrum and milk. In the group of cows immunized with the BRV vaccine, the *R* value for this cytokine in milk (R_{BRV} milk = 11.6) was roughly 4 folds higher than the value determined for the group vaccinated with VP6 (R_{VP6}milk = 3) whilst it remained almost constant for the colostrum (R_{VP6} col. = 1.19; R_{BRV} col. = 1.17). The same pattern was observed with the *R* values for IL-10 in colostrum (R_{VP6} col. = 1.06; R_{BRV} col. = 1.03), which remained constant with respect to *R* value in milk (R_{VP6} milk = 1.06; R_{BRV} milk = 1.17).

The expression of IFN-α mRNA was always higher in the animals immunized with VP6 subunit vaccine (R_{VP6} col. = 0.35; R_{vp6} milk. = 1.82) than in animals immunized with BRV vaccine (R_{BRV} col.=0.17; R_{vBRV} milk. = 0.13).

Table 3
Efficiency Value (E), Efficciency percentage and R^2 value for different cytokines.

	E (efficiency)	% Efficiency	R^2
GAPDH	2.03	103	0.981
IFN-α	1.98	98	0.995
IFN-γ	1.99	99	0.982
IL-4	1.97	97	0.991
IL-6	1.97	97	0.990
IL-10	2.04	104	0.988
IL-12	2.00	100	0.992

4. Discussion

In a previous work we have reported the humoral immune response induced in cows by a VP6 subunit vaccine which was passively transferred to the newborn calves (Gonzalez et al., 2010). In this work we begin to study, through the qPCR technique, the immune cellular response in animals that were immunized with that vaccine. Most investigations has tended to bypass the potential role of macrophages, T and B cells and another colostral components which can be transfer passively to the calf's blood stream. This knowledge about the transference of colostral cytokines and cells from the mother to the offspring provides a basis for the study and the understanding of the immune neonatal system and is important for the design of prophylactic vaccines and for the optimization of therapeutic protocols. Certain aspects of immune mechanisms in cattle and their regulation by cytokines and growth factors have already been addressed (Kehrli and Shuster, 1994; Shuster et al., 1996; Sordillo et al., 1997; Taylor et al., 1997). However, there is still a lack of knowledge in the interplay of the immune components involved in the generation of protective immunity transferred by colostrum. In that sense, this is the first report that analyzes the cytokines present in colostrum and milk from pregnant cows that were immunized with experimental BRV vaccines.

The expression of some cytokines has been historically difficult to detect. However, in this work, we were able to quantify the cytokines mRNA expression by a qPCR method. Cytokine quantification was achieved using the Ct comparative method, and were expressed as "*n*-fold upregulation of cytokine transcription" in relation to a calibrator which is represented by the smallest signal detectable for that specific cytokine. Our study was performed on healthy mammary glands. The samples were obtained at the same time for all groups and we can assume that the n-fold upregulation was only due to the different response against the two vaccines assayed. In that sense, the results for IL-6 (a pro-immflamatory cytokine) suggests that the BRV vaccine could induce higher inflammatory stimulus in comparison with the VP6 vaccine (*R* value BRV higher than *R* value VP6).

Collins et al. (Collins et al., 1998) demonstrated that the IL-12 was able to up-regulate the IFN-γ production in PBMC from bovines infected with the syncicial respiratory virus. Then, we could assume that the presence of IL-12 in colostrum, could be a cause for the presence of IFN-γ transcripts afterward. Furthermore, IFN-γ is a cytokine generated after a viral infection; therefore, it was expected that the presence of IFN-γ transcripts would be exacerbated in cows vaccinated with BRV when compared to cows vaccinated with a subunit vaccine. However, IFN-α is also a cytokine

Table 4
R value for each cytokine in colostrum and milk for different vaccines (VP6 and BRV).

	IL-6		IL-12		IFN-γ		IL-10		IL-4		IFN-α	
	VP6	BRV	VP6	BRV	VP6	BRV	VP6	BRV	VP6	BRV	VP6	BRV
COLOSTRUM	0.55	2.55	0.27	0,84	1.19	1.17	1.06	1.03	ND	Ct > 40	0.35	0.17
MILK (day + 5)	3.90	16.58	ND	ND	3.00	11.60	1.06	1.17	ND	ND	1.82	0.13

Ct > 40: corresponds to the samples that showed no amplification after qPCR assay.
ND: samples in wich the calibrator showed a Ct > 40, therefore the Pfaffl equation could not be calculated.

with a potent immunoregulatory and antiviral activity but when we analyze IFN-α we observed an R value for VP6 vaccine higher than the BRV vaccine.

IL-10 is secreted by Th2 cells among other cells. This cytokine downregulates Th1 proliferation and the cytokines secreted by them (Riollet et al., 2000). The little values for IL-10 could also show a trace of Th2 biased immune response. Unfortunately, we have not been able to determine a *R* value for IL-4, which is a characteristic Th2 cytokine.

Blutt y col. (Blutt et al., 2006) suggested that T cells are essential for the protection induced by a non-replicating vaccine. Furthermore, these cells could be contributing to maintain the long-term immunity, without antibodies, after a rotavirus infection. In that sense, high levels of protection were observed in mice model, when a VP6 linked to MBP (maltose binding protein) was administred with an adequate adjuvant. This protection was found to remain fully intact for at least one year and the CD4 + T-cells were the unique lymphocytes required (Choi et al., 1999; McNeal et al., 2002; VanCott et al., 2006). A possible mechanism for the protection observed could be mediated the direct or indirect effect caused by IFN-γ or IL-17, produced by specific stimulation of CD4 T-cells after virus infection (Smiley et al., 2007).

In summary, the VP6 vaccine used in this study was able to upregulate certain cytokine transcripts that suggested the induction of a Th2 immune response; however, a group of cytokine transcripts associated with Th1 were also expressed. This could be due to the formulation of the VP6 vaccine with an oleic adjuvant, generating an emulsion W/O which could lead to a Th1 immune response. It is also possible that the VP6 vaccine had incorporated CpG residues from the baculovirus lysates, which would have induced a Th1 immune response via TLR9 (Abe et al., 2005).

Namely, it is probably that this subunit vaccine had exerted its function not only by an absolute mechanism but also through a mixed Th1/Th2 immune response. However, an exhaustive analysis at the protein level should be considered for defining a final pathway reached by this VP6 subunit vaccine.

5. Conclusions

The detection of transcripts from cytokines of interest demonstrated that the colostral cellular population was active and that they could exert its action influencing the final immune response in calves. This cellular response could collaborate to the newborn's protection against BRV infection.

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