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Quantification of water buffalo (*Bubalus bubalis*) cytokine expression in response to inactivated foot-and-mouth disease (FMD) vaccine

Claro N. Mingala [a,b], Satoru Konnai [a], Fe A. Venturina [b], Misao Onuma [a], Kazuhiko Ohashi [a,*]

[a] Laboratory of Infectious Diseases, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Hokkaido 060-0818, Japan
[b] Philippine Carabao Center National Headquarters and Genepool, Science City of Munoz, 3120 Nueva Ecija, Philippines

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ABSTRACT

This study describes the quantification of cytokine expression of vaccinated water buffaloes with FMD inactivated vaccine. Using real-time PCR quantification assay, expression of Th1 (IL-2, IL-12p40, IFNγ); Th2 (IL-4, IL-10) and inflammatory (IL-6, TNFα) cytokines were quantified weekly for the entire three-week duration of the experiment. It was noted that IFNγ, IL-10 and TNFα had peaked on week three post-vaccination while the remaining cytokines peaked on the second week and decreased by the third week. The counteraction between IFNγ and IL-4 was noted as well as the possible suppressive action of IL-10 to that of IL-2 and IL-12, which is a common phenomenon between Th1 and Th2 cytokines. Synergy between TNFa and IL-6 was also observed. These findings suggest that within the immune system of water buffalo there is a dynamic cell-mediated and humoral interaction in response to immunogen. This assessment of the cytokine expressions is vital for the study of water buffalo disease progression and concurring protective immune responses.

1. Introduction

The cells of the immune system communicate by a network of soluble proteins called "cytokines", which are products of the respective cells of the immune system. These cytokines form a network of bioactive agents, which synergize or antagonize each other's effects. Th1 and Th2 cytokines are the two major types that significantly contribute to the combating of pathogens. The balance of these cytokines could determine the disease outcome for a susceptible animal. Cytokines are extremely crucial to immune response in an animal; therefore, explicating the role and expression of these protein molecules is particularly vital. Unfortunately, water buffalo immunology research at the molecular level has been very limited.

Recently, a large number of assays, which are mostly protein-based tests such as ELISA, Elispot and Flowcytometry, have been introduced in order to measure cytokine expressions for various animal species. The other tests were PCR based, which rely on the analysis of expression kinetics through electrophoresis reading. In water buffalo, cytokine expression reports were already published mainly using the latter procedure given that protein-based assays have their own limitations due to unavailability of specific antibodies (Premraj et al., 2005, 2006a,b,c).

Previous studies on water buffalo cytokine expression were carried out by measuring the changes of cytokine level in scheduled time of incubation stimulated with mitogen in vitro (Premraj et al., 2005, 2006a,b). Interesting findings were reported on the dynamics shown by water buffalo cytokines in a controlled environment. Meanwhile, previous studies also presented the current trends in quantifying gene expressions using real-time PCR. This has been developed as a simple and rapid quantification of several genes. Different animals were already tested using this assay in measuring their cytokine expressions (Konnai et al., 2003; Odbileg et al., 2005).

In the present study, cytokine expression was analyzed in situ. This study attempts to describe and elaborate on the cytokine responses of water buffalo after vaccination. Since Aftofor FMD vaccine is the regular vaccine used in the farm it was decided to use this as the test immunogen to establish the actual scenario, avoid any measurement bias in the result and prevent the risk of introducing a new vaccine in the farm, which might cause vaccine outbreak. This study will certainly create independent information from that of cattle upon which, at present, the decisions and designing of immunization schemes for water buffaloes are mostly based. There were various factors to be considered since the experimental animals used were contained in a typical farm management system. While the health condition of the animals was apparently normal, the stress caused by animal handling was unavoidable. Nevertheless, the outcome of this study significantly confirmed the usual cytokine expression in response to

* Corresponding author. Tel.: +81 80 5588 4267; fax: +81 11 706 5217.
E-mail address: okazu@vetmed.hokudai.ac.jp (K. Ohashi).

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stimulation. This was the first time that water buffalo cytokine expression was described and quantified by the use of real-time PCR quantification assay.

2. Materials and methods

2.1. Experimental animals, vaccination and blood collection

Twelve seemingly healthy riverine type water buffaloes were used as experimental animals. They were all between 6 months and 1 year old during the experiment. Six of them were used as treatment while the other half was used as control. The treatment animals were vaccinated with Aftofor monovalent, inactivated FMD vaccine in double-oil-emulsion (DOE). This vaccine is specifically developed and produced for the Philippines, which contains "O 1 Philippine strain". Each treatment animals were given 1 mL of the vaccine intramuscularly as recommended by the manufacturer. Another 1 mL dose was administered for the succeeding booster shots. Heparinized whole blood was collected from all of the animals at day 0 (pre-vaccination) and subsequently one, two and three weeks post-vaccination.

2.2. Primers

Oligonucleotide primers for Th1 (IL-12p40, IFNγ, IL-2), Th2 (IL-4, IL-10) and inflammatory (IL-6, TNFα) cytokine genes and the β-actin gene as an internal control were designed from GenBank sequence information. Based on the sequences of bubaline cytokine genes, primers were designed. The details of all oligonucleotide primer sequences, primer lengths, predicted product lengths, and optimal amplification conditions are listed in Table 1.

2.3. PBMC isolation, RNA extraction and RT-PCR

Peripheral blood mononuclear cells (PBMCs) were purified by density gradient centrifugation on Percoll (Amersham-Pharmacia) from heparinized venous blood of water buffaloes. Total cellular RNA was extracted from cells by using TRIzol reagent (Invitrogen) according to manufacturer's instruction. Synthesis of cDNA was carried out as described elsewhere (Mingala et al., 2006).

2.4. Real-time PCR

Real-time PCR was performed using a Light cycler, (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Real-time PCR using SYBR Premix Ex *Taq* (Takara) was performed by a standard protocol recommended by the manufacturer with 3 μL of cDNA, which was added to a 17 μL reaction mixture including 0.4 μL of 10 pmol of each primer and 10 μL of SYBR Premix Ex *Taq* and DDW, and run in a Light cycler under the following conditions: at 95 °C for 1 min as the denaturation program, followed by 45 cycles of 94 °C for 1 s, each of optimal annealing temperature for 5 s, 72 °C for 10 s, and each of optimal fluorescence measurement temperature for 1 s as the amplification and quantification programs, 60–99 °C with a heating rate of 0.1 °C/s as the melting curve program, and finally cooling to 40 °C as the cooling program. The optimal conditions for each cytokine amplification and quantification were summarized in Table 1. Real-time amplified PCR products were sequenced and 100% homology to the bubaline cytokine sequences was confirmed. Moreover, to standardize and estimate mRNA expression, calibration curves were made from the measured fluorescence of dilution series of the control cDNA to create the same amplification curves. The results were shown as the ratios obtained by dividing the concentrations of the PCR products from the cytokine mRNAs by the dose from the β-actin mRNA as described previously. (Konnai et al., 2003; Odbileg et al., 2005)

2.5. Liquid-phase blocking ELISA

Expression of target cytokines was also checked with the humoral response of the animals. A liquid-phase blocking (LPB) ELISA was performed independently by FMD taskforce of the Philippine Animal Health Center (PAHC), which is supported by the FAO FMD surveillance program.

2.6. Statistical analysis

All statistical analyses were performed using GraphPad Software online version (www.graphpad.com/). Data were analyzed by one-way ANOVA and unpaired *t*-test. Differences between groups were considered significant if probability values of $p < 0.05$ were obtained.

3. Results

After each treatment, cytokines (as mentioned above) were analyzed by real-time PCR. These findings revealed that all of them were upregulated. The bar graphs (Figs. 1 and 2) that were generated to compare the expression of each cytokine showed that IL-2 immediately increased ($p < 0.05$) on the first week post-vaccination compared to the rest of the cytokines. Interestingly, IFNγ, IL-

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

Target gene	Primer set (5′-3′)	Primer length (bp)	Product length (bp)	Annealing temperature (°C)
β-actin	F – CGC ACC ACC GGC ATC GTG AT	20	227	60
	R – TCC AGG GCC ACG TAG CAG AG	20		
TNFα	F – TAA CAA GCC GGT AGC CCA CG	20	218	60
	R – GCA AGG GCT CTT GAT GGC AGA	21		
IL-6	F – CTG CAA TGA GAA AGG AGA TA	20	192	55
	R – GGT AGT CCA GGT ATA TCT GA	20		
IL-2	F – TTT TAC GTG CCC AAG GTT AA	20	293	58
	R – GAG GCA CTT AGT GAT CAA GTC	21		
IFNγ	F – GTC TCC TTC TAC TTC AAA CT	20	255	55
	R – ATT CTG ACT TCT CTT CCG CT	20		
IL-12p40	F – CAG GGA CAT CAT CAA ACC AG	20	214	58
	R – CTT GTG GCA TGT GAC TTT GG	20		
IL-4	F – CAA AGA ACA CAA CTG AGA AG	20	121	53
	R – AAG CTG TTG AGA TTC CTG TC	20		
IL-10	F – CTG CTG GAT GAC TTT AAG GG	20	187	60
	R – AGG GCA GAA AGC GAT GAC AG	20		