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Differential expression of toll-like receptor 9 by various immune compartments of buffalo

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Toll-like receptor 9 (TLR9) has been characterized as a receptor that recognizes unmethylated CpG (2'-deoxy-ribocytidine-phosphate-guanosine) motifs present in viral and bacterial DNA. Evidence from both *in vitro* and *in vivo* studies confirm that CpG-ODN can activate the immune system in variety of domestic species including cattle, sheep, pigs, horses, dogs, cats and chicken (Brown *et al*. 1998, Kamstrup *et al*. 2001, Zhang *et al*. 2001, Mena *et al*. 2003, Nichani *et al*. 2007). The flanking sequences in synthetic CpG oligodeoxynucleotide (ODN) and the cellular pattern of TLR9 expression can influence species-specific responses to CpG-ODN (Griebel *et al*. 2004). The cellular pattern of TLR9 expression and recruitment of specific cell populations may be important factors contributing to interspecies differences in innate and adaptive immune responses following CpG-ODN stimulation (Griebel *et al*. 2005).

Proper targeting of CpG ODN for the induction of innate immune defenses requires an understanding of immune compartments which express high abundance of TLR9. Buffalo is economically important animal in Asia, some European and South American countries. It is mainstay of dairy husbandry and contributes significantly to the milk and meat production in India. It is pertinent to devise effective strategies for prevention and control of buffalo diseases. There is a little information on the differential expression of TLR9 in buffaloes *(Bubalus bubalis)*. Therefore, the present study was carried out to perform quantitative analysis of TLR9, in different immune compartments of buffalo calves.

Tissue and blood samples

Blood samples were collected aseptically from the external jugular vein of buffalo calves by venipuncture in 15 ml sterile centrifuge tubes containing 0.5 ml of 7.5% ethylene diamine tetra-acetic acid (EDTA) as anticoagulant. A variety of lymphoid (mesenteric lymphnode, tonsil and spleen) and mucosal tissues (lung and intestine) of healthy buffalo calves were collected immediately after their sacrifice from slaughter house, New Delhi. The tissues were cut into slices of less than 0.5 cm thickness and were stored in RNA later till further use.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation on Histopaque as per manufacturer's instructions. The cells were washed thrice with PBS by centrifuging at 200 × *g* for 10 min at room temperature. The viable cells were counted by trypan blue dye exclusion technique, resuspended at 10^7 cells per ml in PBS and used for RNA extraction.

RNA extraction

Total RNA was extracted from the tissues homogenized by homogenizer (Polytron 2100) as well as from $1x10^7$ PBMCs using commercial kit, as per manufacturer's instructions. The quality and quantity of RNA was determined by spectrophometer by taking the absorbance at 260 and 280 nm. The RNA samples in 1µg quantity were treated with DNase I in order to remove genomic DNA as per manufacturer's instructions. The integrity of DNase treated RNA samples were again determined by spectrophotometry. The RNA samples having A_{260}/A_{280} ratios from 1.9 to 2.1 were used in further experiments.

Relative quantitation of TLR9 by qRT-PCR

Quantitative RT-PCR was performed on DNase-I treated RNA samples from different tissues as well as PBMCs using one step RT-PCR kit. The reagents were optimized to determine the minimum primer and probe concentrations giving the maximum Rn (the difference in total fluorescence between a reaction containing all components and an unreacted control). A housekeeping gene *GAPDH* was used as an endogenous control for normalization of experimental results using specific primers as described earlier (McGuire

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Table 1. Primers for quantitative RT-PCR

Gene	Primer	Sequence 5'-3'	Amplicon length (bp)
TLR9	Forward	CAACAGCATCTCGCAGGC GGTTAAT	100
	Reverse	CATGGTACAGGTCCAGCTTGT TGTG	
GAPDH	Forward	TTCTGGCAAAGTGGACATCGT	112
	Reverse	CTTGACTGTGCCGTTGAACTTG	

et al. 2004). Primers used for qRT-PCR are listed in Table 1. Quantitative RT-PCR for GAPDH and TLR9 was performed in 25 µl reactions with Green *Taq* ReadyMix 1 unit/µl MuLV, 100 ng of total RNA and 600 nM final primer concentration for each forward and reverse primer for GAPDH and TLR9, 1x ROX was used as reference dye using a Bio-Rad Miniopticon Real Time PCR detection system. Thermal cycling conditions were 3 min at 95°C and 45 cycles of 15 sec at 95°C and 45 sec at 60°C. The real time PCR specificity was also confirmed with melting curve analysis. The threshold cycle numbers (C_t) were automatically determined by the PCR detection system. Each experiment was performed in duplicate. Each sample was amplified for GAPDH and TLR9 simultaneously in the same plate to avoid plate to plate variations.

TLR9 mRNA expression was measured by relative quantification, which compared the threshold cycle (C_t) of the sample of interest to the C_t generated by a reference sample by $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001). When template DNA concentration was plotted against the DC_t, it revealed approximately equal amplification efficiencies for GAPDH and TLR9.

The targeting of TLR9 has emerged as a powerful tool in the generation of Th1 adaptive immunity, and has shown promise for enhancing the efficacy of vaccination (Griebel *et al*. 2005). The expression of TLRs is not restricted to cells that are involved in antigen processing and presentation but also has been detected in a wide range of tissues (Iqbal *et al*. 2005). TLR9 expression in different tissues has been reported in buffaloes (Vahanan *et al*. 2008). The quantification of expression is essential to know the abundance of TLR9 in various immune compartments. In the present study, the transcriptional expression of TLR9 was quantified in lymphoid, blood and mucosal tissues of buffalo.

Before proceeding for qPCR, the specificity of primers for gene expression was studied in *Bubalus bubalis.* All cDNAs prepared by reverse transcription of 1 µg of total RNA of different tissues expressed detectable levels of GAPDH and TLR9 mRNA. The electrophoretic separation of the PCR products obtained from the amplification of cDNAs with primers designed for real time PCR for GAPDH and TLR9 revealed products of expected size of 112 bp and 100 bp respectively.

Amplification of both the genes was performed in the same plate and the plates were repeated twice. TLR9 mRNA expression was measured by relative quantification, which compared the threshold cycle (C_t) of all the tissues as well as PBMCs to the C_t generated by lymphnode (showing the highest TLR9 expression) by $2^{-\Delta\Delta Ct}$ method. A housekeeping gene GAPDH was used as an endogenous control for normalization of experimental results. Overall, diverse features of variation in TLR9 gene expression patterns among different tissues were seen. The highest quantitative expression of TLR9 was exhibited by lymphnode, followed by spleen, tonsil, lung, intestine and PBMC taking GAPDH as housekeeping gene (Fig. 1).

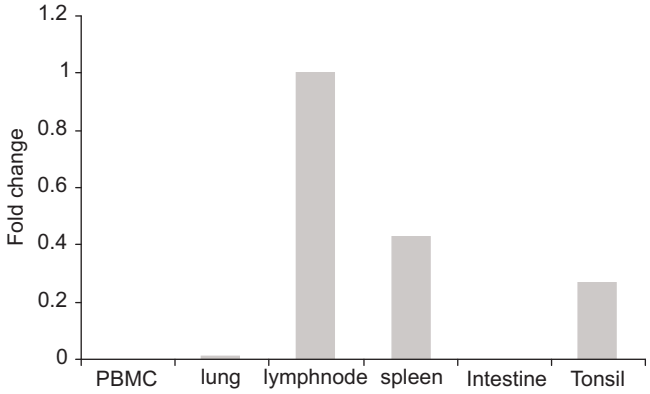

Fig. 1. Quantification of TLR9 mRNA expression by different tissues as determined by Real-time PCR using $2^{-\Delta\Delta Ct}$ method with respect to lymphnode.

All these findings together revealed high level of expression in lymphoid tissues and relatively low expression in blood and mucosal tissues. This result is in conformity with expression of TLR9 in several lymphoid tissues from four cats (Ignacio *et al*. 2005) and cattle (Griebel *et al*. 2004). In our study we have shown that lungs exhibited lower abundance of TLR9 than that of lymphoid tissues. Similar results have been reported for cattle (Griebel *et al*. 2004). Alveolar macrophages do not express appreciable quantities of TLR9 in the resting state in mice, however, it has been observed that lung dendritic cells and B cells highly expressed TLR9 mRNA and responded to CpG-ODN (Sparwasser *et al*. 1997). Our investigations show that overall abundance of TLR9 in lungs is comparatively low.

SUMMARY

Differences in the pattern of gene expression among

lymphoid, mucosal and blood has been established. High level of expression in lymphoid tissues and relatively low expression in blood and mucosal tissues were observed. Thus, our increasing understanding of the buffalo TLR9 system could provide the molecular basis for targeting its agonist in host system.

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