Bacterial DNA Induced TNF-α Expression in Buffaloes (Murrah) in Comparison to that of Cross Breed Cattle

P. Nisha[1], J. Thanislass[1,*], P.X. Antony[2], H.K. Mukhopadhyay[2] and K.V. Subba Reddy[1]

[1]*Department of Veterinary Biochemistry, Rajiv Gandhi College of Veterinary and Animal Sciences, Puducherry-605009, India*

[2]*Department of Veterinary Microbiology, Rajiv Gandhi College of Veterinary and Animal Sciences, Puducherry-605009, India*

Abstract: Buffaloes are generally considered to be disease resistance. But systematic studies to understand the underlying mechanism of disease resistance in buffaloes in comparison to that of cattle are scanty. Therefore, the present study was undertaken to study the immune response in terms of TNF-α expression in PBMCS isolated from buffaloes in comparison to that of cattle. PBMCs were isolated from blood collected from healthy buffaloes and cross breed cattle and incubated with bacterial (*E. coli*) DNA at different concentration for a different period of time. Total RNA was isolated and mRNA expression of TLR9 and TNF-α was studied. Expression of actin gen was studied as positive control. Incubation of PBMCs with bacterial DNA resulted in the expression of TLR9 in both, buffaloes and cattle. But, the expression of TNF-α was seen only in the case of buffaloes and the level was found to increase with the increase in bacterial DNA concentration and time. Thus this study reports the inherent difference in the immune response of buffaloes in comparison to that of cattle.

Keywords: *Bubalus bubalis*, Cattle, Immune response, TLR9, TNF-α, Cytokines, Buffalo, Actin, Bacterial DNA, Immunotherapy.

INTRODUCTION

Water buffalo and cattle are susceptible to similar spectrum of infectious agents, but water buffalo is found to respond differently to certain infections like *Fasciola gigantica* [1] and *Brucella abortus* [2]. Wallowing behavior exposes buffalo to water borne pathogens which is not normally encountered by the cattle. But, the management of buffaloes, including health management is same as that of cattle which may not find answer to all the problems of buffaloes. Therefore, it is necessary to undertake systematic studies specific to buffaloes which will, not only contribute to the improvement in the management of water buffaloes and also provide insight into the mechanisms accounting for the differences in disease resistance because buffaloes are generally considered to be disease resistance. To evaluate the immune responses of water buffalo to infectious agents and for the development of potential vaccines, it is necessary to characterize the immune system of water buffalo and elucidate the changes in the immune response that account for the development of protective immunity [3]. Characterization of Toll like receptors will be one of the approaches to understand the underlying principle of immune response in buffaloes.

*Address correspondence to this author at the Department of Veterinary Biochemistry, Rajiv Gandhi College of Veterinary and Animal Sciences, Puducherry-605009, India; Tel: +919443860053; Fax: +91-413-2272005; E-mail: jthanislass@gmail.com

Toll-like receptors (TLRs) are part of the innate immunity, can recognize conserved pathogen-associated molecular patterns (PAMPs) through TLRs of immune cells [4-6]. Recognition of invading pathogens then triggers cytokine production and up-regulation of co-stimulatory molecules in phagocytes, leading to the activation of T cells. Thus, TLRs play a pivotal role in linking the innate immunity with the acquired immunity.

In our laboratory, the expression of TLRs in different tissues of buffalo was demonstrated [7] and characterized TLR signaling process in buffaloes in comparison with that of cattle [8]. We also reported nucleotide variations in TLR4 gene corresponding to functional domain of protein in buffaloes [9]. The present study reports the TNF-α mRNA expression in buffaloes in comparison to that of cattle when PBMCs were incubated with bacterial (*E. coli*) DNA which is usually recognized by TLR9 of immune cells.

MATERIALS AND METHODS

The blood (10 ml) was collected from healthy buffaloes (Murrah) and cross breed cattle of 3 to 4 years of age, five animals each, using heparin coated vacutainer. Peripheral Blood Mononuclear Cells (PBMCs) were isolated from blood using Ficoll-Paque (Sigma Aldrich) Density Gradient centrifugation. The PBMCs thus isolated were incubated with bacterial (*E. coli*) DNA (1.0 µg and 0.5 µg), isolated from

ISSN: 1927-5196 / E-ISSN: 1927-520X/13

Name of the Gene	Primer	Expected Size of the Product
TLR9	Forward: 5'GCACCTGTCGCTCAAGTACA3'	446 bp
	Reverse:5'CGAAGGACACCTTCTTGTGG3'	
TNF-α	Forward:5'AACGGCGTGAAGCTAGAAGA3'	354 bp
	Reverse:5'GGCGATGATCCCA AAGTAGA3'	
Actin(Positive Control)	Forward:5'GACAATGGTTCTGGCATGTG3'	228 bp
	Reverse:5'CCAGATCCTCTCCATGTCGT3'	

E*scherichia coli* (MTCC 40, Chandigarh) culture by following the method of Dalpke *et al.* [10]. The quality of DNA isolated was found to be good as indicated by the ratio of absorbance (1.8) at 260nm and 280nm (Hitachi Spectrophotometer). The cells were incubated with *E. coli* DNA in the presence of lipofectamine which was used to enhance the uptake of DNA, for a period of two and four hour's duration in a CO_2 incubator at 37°C and 5% CO_2. Similar preparation of PBMCs subjected for incubation without the *E. coli* DNA but with lipofectamine which served as control. The experiments were repeated thrice.

At the end of the incubation period, total RNA was isolated using Tri reagent (Sigma-Aldrich) and was used to check the mRNA expression of TLR9, TNF-α and Actin (Postive control) using following primers in RT-PCR.

cDNA was synthesized using AMV reverse transcriptase system [11] and PCR amplification was carried for 30 cycles. The annealing temperature used was 55° C for actin and 56° C for TLR9 and TNF-α. The standard PCR conditions of primary denaturation at 94° C for 2', 30 cycles of denaturation at 94° C for 45 sec., annealing at respective temperature, 55ºC(Actin), 56ºC(TLR9 and TNF-α), for 45 sec.,, and elongation at 72° C for 45 sec., final elongation at 72° C for 2 minutes were followed. The amplified products were checked on 2% Agarose gel electrophoresis.

As these primers were designed based on the cattle sequence (TNF-α - Accession no.AY221123; Actin-Accession no.BC102992; TLR9-Accession no.BTA509824) using primer3 [12], the specificity of amplification with respect to cattle and buffalo DNA was tested. The primers were found to amplify the DNA from the both species with equal efficiency. Further, *in silico* examination revealed the specificity of primers towards the respective genes belong to both the species of animals.

RESULTS AND DISCUSSION

Incubation of PBMCs from cattle and buffalo with *E. coli* DNA resulted in the expression of TLR9 (Figure **1**), but not in the case of cells incubated with lipofectamine alone without the bacterial DNA. The level of expression was almost equal in cattle and buffalo. The expression of actin gene was used as positive control (Figure **2**).

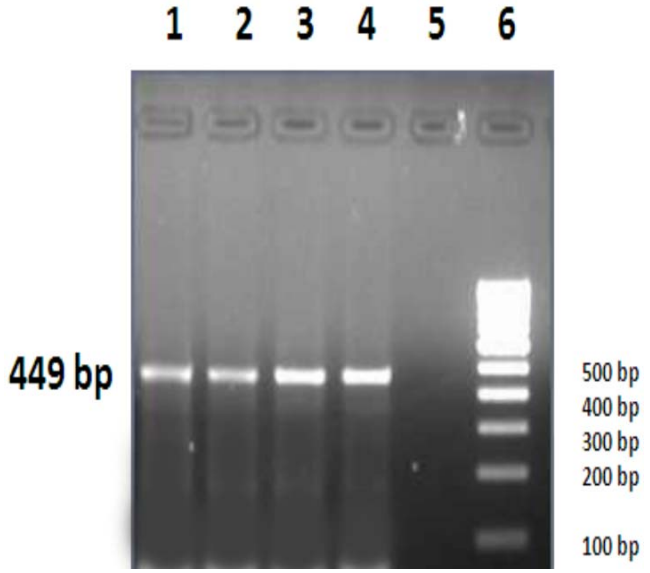

Figure 1: RT-PCR for TLR9 expression. (RNA isolated from the following and used for RT-PCR analysis. Lane 1 & 2: PBMC (Cattle) incubated with *E. coli* DNA(1.0 µg) for a period of 4 hours; Lane 3 & 4: PBMC (Buffalo) incubated with *E. coli* DNA(1.0 µg) for a period of 4 hours; Lane 6: 100 bp DNA ladder).

Among the different types of TLRs, TLR9 is found to recognize bacterial DNA as well as viral DNA containing unmethylated CpG dinucleotides [13, 14]. DNA isolated from bacterial species is found to activate TLR9 and the extent of expression is found to depend on CpG content of the DNA [10]. Generally, it is observed that rather high concentration of DNA is necessary to stimulate TLR9 [15]. But the use of lipofectamine enabled the expression of TLR9 even with nanogram quantity of DNA. Cytokines such as tumor necrosis factor (TNF-α), interleukins-1β and IL-6 are often held to be the principal exemplars of the panel of pro-inflammatory mediators produced in response to TLR stimuli [16, 17]. In the present study also incubation of PBMCs with bacterial DNA resulted in the mRNA expression of TNF-α but it was

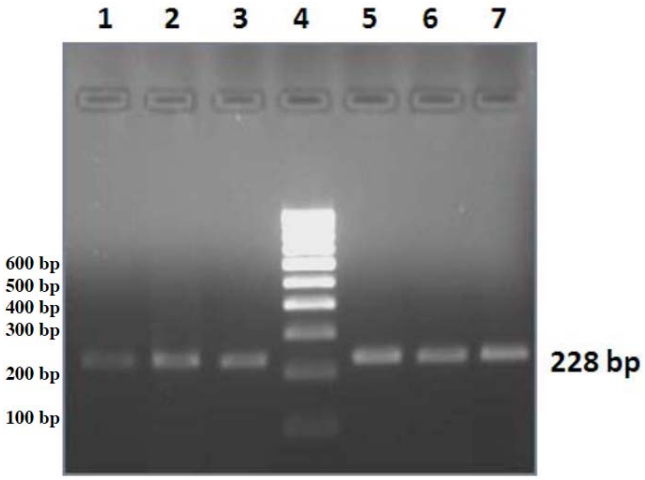

Figure 2: RT-PCR for Actin gene expression. (RNA isolated from the following and used for RT-PCR. Lane 1: PBMC (Cattle) Incubated without *E. coli* DNA; Lane 2: PBMC (Cattle) Incubated with *E. coli* DNA (1.0 µg) and lipofectamine for a period of 2 hours; Lane 3: PBMC (Cattle) Incubated with *E. coli* DNA (1.0 µg) and lipofectamine for a period of 4 hours; Lane 4: 100 bp DNA ladder; Lane 5: PBMC (Buffalo) Incubated without *E. coli* DNA; Lane 6: PBMC (Buffalo) Incubated with *E. coli* DNA (1.0 µg) and lipofectamine for a period of 2 hours; Lane 7: PBMC (Buffalo) Incubated with *E. coli* DNA (1.0 µg) and lipofectamine for a period of 4 hours).

significantly seen only in the case of buffalo but not in cattle (Figure **3A**, **B**).

The level of TNF-α expression in buffalo was found to increase with the increase in concentration of *E. coli* DNA and incubation time. Zhang *et al*. [18] reported that PBMCs, monocytes and macrophages isolated from cattle and incubated with CpG oligonucleotides secreted low level or undetectable levels of TNF-α. Similarly, Weiss *et al*. [19] reported that monocytes isolated from *Mycobacterium avium subsp paratuberculosis* infected cows had low expression of TNF-α when co-incubated with MAP. We also reported enhanced level of expression of TNF-α in PBMCs isolated from buffaloes when incubated with LPS in comparison to that of cattle [9]. Recently, Mingala *et al*. [20] reported that promoter region of TNF-α gene in swamp type water buffalo had higher transcription activity compared to riverine water buffalo. Therefore it may concluded that TNF-α expression seen in buffalo (swamp type) used in this study could be due to the presence of strong promoter region of TNF-α gene which would have enabled the induction of TNF-α expression even at the use of nanogram quantity of bacterial DNA, but this is not the case in cattle which had already been reported to be poor in TNF-α expression. Thus this study, once again clearly demonstrates the inherent difference in the expression of cytokines by the buffalo in comparison to that of cattle. This difference seen is significant because the TNF-α is known to be associated with inflammation related side effects when compared with the other types of cytokines, this need to be considered at the

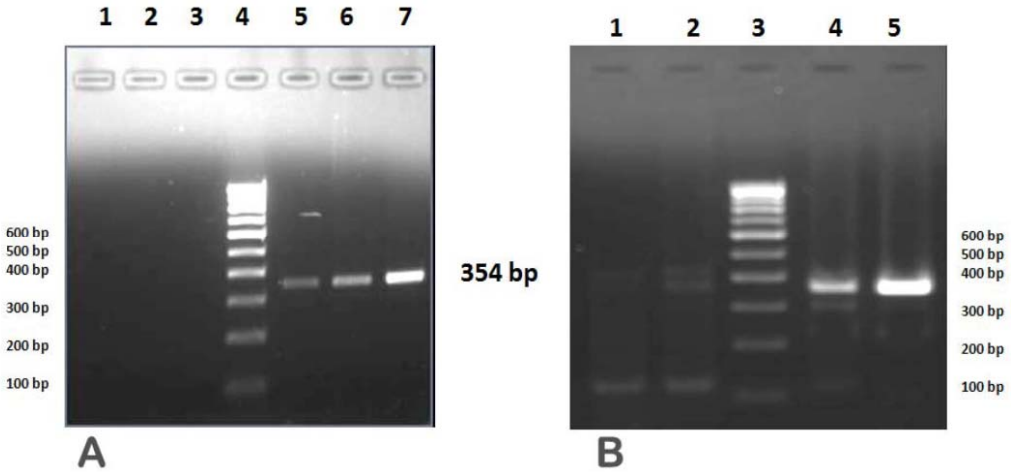

Figure 3: (A). RT-PCR for TNF-α expression. (RNA isolated from the following and used for RT-PCR. Lane 1: PBMC (Cattle) Incubated without *E. coli* DNA; Lane 2: PBMC (Cattle) Incubated with *E. coli* DNA (0.3 µg) and lipofectamine for a period of 2 hours; Lane 3: PBMC (Cattle) Incubated with *E. coli* DNA (0.3 µg) and lipofectamine for a period of 4 hours; Lane 4: 100 bp DNA ladder; Lane 5: PBMC (Buffalo) Incubated without *E. coli* DNA; Lane 6: PBMC (Buffalo) Incubated with *E. coli* DNA (0.5 µg) and lipofectamine for a period of 2 hours; Lane 7: PBMC (Buffalo) Incubated with *E. coli* DNA (0.5 µg) and lipofectamine for a period of 4 hours).

(B). RT-PCR for TNF-α expression. (RNA isolated from the following and used for RT-PCR. Lane 1: PBMC (Cattle) Incubated with *E. coli* DNA (1.0 µg) for a period of 2 hours; Lane 2: PBMC (Cattle) Incubated with *E. coli* DNA (1.0 µg) for a period of 4 hours; Lane 3: Molecular weight marker; Lane 4: PBMC (Buffalo) Incubated with *E. coli* DNA (1.0 µg) for a period of 2 hours; Lane 5: PBMC (Buffalo) Incubated with *E. coli* DNA (1.0 µg) for a period of 4 hours).

time of deciding the immuno-therapeutic regimen for the buffaloes.

ACKNOWLEDGEMENTS

The financial assistance (Ref.BT/PR4729/AAQ/01/180/2004) is provided by the Dept. of Biotechnology, Ministry of Science & Technology, Govt. of India.

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Received on 15-10-2012 Accepted on 10-12-2012 Published on 22-03-2013

DOI: http://dx.doi.org/10.6000/1927-520X.2013.02.01.2