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Bacterial Lipopolysaccharide Activates Toll-Like Receptor 2 and Toll-Like Receptor 4 Gene Expression in PBMC of Vechur Cattle

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Abstract

Vechur cattle, an indigenous breed of Kerala and it is the smallest cattle breed in the world. They are highly disease resistant. The occurrence of mastitis is very Rare in this breed as compared to crossbred cattle. Rearing of these Vechur breed is more cost effective as they require less feed. Therefore, characterisation of factors involved in the immune system of these breeds might provide an insight into the mechanisms involved in the variation in disease resistance. Toll-like receptors (TLRs) are part of the innate immunity, can recognize the particular pathogens through Pathogen Associated Molecular pattern s (PAMPs) and play important roles in host defense. *TLR2* and *TLR4* important TLR mediate the responsiveness to bacterial lipopolysaccharide (LPS). Since Vechur cattle are less susceptible to mastitis, *in vitro* expression assay of *TLR*s were accessed by challenging the Peripheral Blood Mononuclear Cells (PBMC) with bacterial *LPS.* Treatment of PBMC with LPS, significantly increased *TLR2* and *TLR4* genes expression ($p \leq 0.01$) in Vechur cattle breed when compared with that of control and crossbred cattle. Among the two *TLR*s studied, the relative expression of mRNA in Vechur cattle was relatively higher for *TLR2* (6.90) than *TLR4* (4.24). The higher expression of *TLR 2* and *TLR 4*might contribute maximum innate immune response against the mastitis bacteria in Vechur cattle.

Keywords: TLRs, gene expression, LPS, PBMC, vechur

1. Introduction

Mastitis is the most prevalent and one of the economically imposing diseases in dairy cattle, commonly developed in response to intramammary bacterial infection (Seegers et al., 2003; Zuhair, 2017). Vechur cattle, a rare breed of *Bos indicus*, are an indigenous breed of Kerala state of India had shown less susceptibility to mastitis as compared to crossbreed cattle. Characterisation of factors involved in the immune system of Vechur breeds might provide an insight into the mechanisms involved in the variation in disease resistance. Toll-like receptors (TLRs) are part of the innate immunity, can recognize conserved pathogen associated molecular patterns (PAMPs) which is expressed on invading pathogens (Walletet al., 2018; Lemaitre et al., 1996; Aderema and Ulevitch, 2000; Akira et al., 2001), and provoke cytokine production

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and upregulation of co-stimulatory molecules in phagocytes, leading to the stimulation of T cells (Jianet al., 2016). Thus, TLRs play a pivotal role in linking the innate immunity through specific ligand binding of LPS which in turn promote antigen-specific acquired immunity as well (Vidya et al., 2018)

Bacterial lipopolysaccharide (LPS) is a complex glycolipid endotoxin composed of a hydrophobic domain known as lipid A and a hydrophilic polysaccharide region important for most of the LPS-induced biological effects (Schletter et al., 1995; Yang et al., 1998; Weishan et al., 2017). LPS stimulates host cells such as monocytes, macrophages, and B cells through the activation of protein kinases and transcription factors. LPS recognition the key event in host antimicrobial defense reaction (Schletter et al., 1995; Ulevitch and Tobias, 1995; Anna et al., 2021). Among the mammalian TLRs, *TLR2* had shown to mediate the signals of bacterial constituents, including lipoproteins, lipoteichoic acid, and lipopeptides, whereas *TLR4* is found the major and predominant receptor for as at least some types of LPS, whereas *TLR2* is expendable (Dziarski et al., 2001; Phileman et al., 2019).

In present *in vitro* experiment, the relative expression of *TLR2* and *TLR4* genes were accessed in Vechur cattle breed along with crossbreed cattle by challenging the PBMC with bacterial LPS.

2. Materials and Methods

2.1. Experimental period and location

The experiment was carried out during the year 2016 at College of Veterinary and Animal Sciences, Mannuthy Thrissur, Kerala.

2.2. Collection of PBMCs and incubation

The blood samples (10 ml) were collected from three healthy Vechur and crossbreed cattle of 3 to 4 years of age using EDTA coated vacationer. PBMCs were isolated from blood using Ficoll-Paque (Sigma Aldrich) density gradient centrifugation. The cells were cultured in 8 ml RPMI-1640 medium in tissue culture flasks by incubating in a CO_2 incubator at 37°C for 3 hours. During the incubation period 80% RH in an atmosphere of 5% CO_2 was maintained. After incubation, the medium was discarded along with the dead floating cells. The live monocytes cells which were anchored were used for the study.

2.3. LPS induction of PBM cells

The isolated monocytes cells were incubated with the *E.coli* LPS (100 ng ml^{-1}) for 2 hours in a CO_2incubator (5% CO_2 pressure) at 37°C (80% RH). Similar preparation of cells was subjected for incubation without the LPS, which served as control. At the end of the incubation period, the cells were harvested, and total RNA was isolated from the incubated samples (Thanislass et al., 2009)

2.4. RNA isolation and reverse transcription

Total RNA was isolated from PBMC by using TRIzol reagent (Sigma). The quality and quantity of extracted RNA was determined by agarose gel electrophoresis and NanoDrop Spectrophotometer (Thermo Scientific, USA) DNA contamination was removed from extracted total RNA by treatment with DNase 1.

2.5. Complementary DNA (cDNA) synthesis

First strand cDNA was synthesized from isolated RNA using RevertAid first strand cDNA synthesis kit (Thermo Scientific, K1622). The reactions were set up in 0.2 ml PCR tubes. Template RNA 5µg, Oligo (dT) 18 primer 1.0 µl. 5X reaction buffer 4.0 µl, Ribolock 1 µl, dNTP mix (10 mM) 2.0 µl, Revert Aid 1.0 µl and Nuclease free water. The contents of the tube were mixed gently and centrifuged briefly. For RT-qPCR, oligo (dT) 18 primer were used for cDNA synthesis, the reaction mix was incubated for 60 minutes at 42°C. The reaction was terminated at 70°C for 5 minutes.

2.6. Primers synthesis

Primers used for RT-qPCR of *TLR2*, *TLR4* and β-actin were designed using Primer 3 software from bovine mRNA sequence available in the NCBI database (Table 1). β-actin gene was selected as internal control gene.

Table 1: Primer sequence for *TLR2*, *TLR4* and β-actin genes used in RT-qPCR

Name	Sequence (5'→3')	Product size
TLR2 –RT-F	AGCGAGTGGTGCAAGTATGA	114 bp
TLR2 –RT-R	CTGGGGAATGGCCTTCTTGT	
TLR4 –RT-F	GCCGTGGAGACAAACCTAGT	138 bp
TLR4 –RT-R	CTCCAGGTTGGGCAGGTTAG	
β-actin-RT-F	CCACACCTTCTACAACGAGC	105 bp
β-actin-RT-R	ATCTGGGTCATCTTCTCACG	

2.7. PCR Standardization and RT-qPCR

The PCR was carried out in volume of 12.5 µl in 0.2 ml PCR tubes. The PCR was standardized for different gradients of temperatures. For RT-qPCR analysis, Maxima SYBER Green Master Mix (2X) with ROX (Thermo Scientific) were used and RT-qPCR reactions were performed using RT-qPCR system (ECO software). The qPCR mix was in a final volume of 12.5 µL contained 6.25 µl of Maxima SYBER green master mix, 1µl each of forward and reverse primer, 1 µl of Template cDNA and 4.25 µl of nuclease free water. The thermal profile included initial denaturation at 95°C for10 min, this was followed by 40 cycles of denaturation at 95°C for 30s, annealing at 58°C for 20 s, extension at 72°C for 30s. A melt curve of the amplified products was acquired after the 40 cycles by a further run of 95°C for 15 sec, 55°C for 15 sec followed by 95° C for 15 sec to confirm the specificity of the amplified and absence of primer dimer. The $2^{-\Delta\Delta CT}$ method (Kumar et al., 2012).was used to determine the fold change of gene expression level.

3. Results and Discussion

Toll-like receptors are pledged for recognizing cognate ligands

of pathogens such as LPS, resulting in the production of inflammatory responses that defend the host against invading pathogens (Salwa et al., 2019). The major receptors *TLR2* and *TLR4* exhibit different specificities in the recognition of cell wall component of the bacteria. Although *TLR2* functions as a receptor for certain exceptional LPS species (Dixon and Darveau, 2005) *TLR4* is the primitive receptor for Gram-negative bacterial LPS and plays a prominent role in defense against infections (Takeuchi et al., 1999; Theodora et al., 2019). Since *TLR2* and *TLR4* are the best competitors for bridging from the innate immunity of macrophages to the adaptive immunity of T and B lymphocytes, an understanding of their TLRs transcriptional expression and regulation in native macrophages in response to gram-negative bacteria is important (Bohan et al., 2019). In this study, we have explored the expression pattern of presumptive LPS signalling receptors *TLR2* and *TLR4* in PBMC of Vechur and Crossbred cattle. PBMC consists of various inflammatory cell populations that circulate between the vascular system and tissues and are likely to reflect disease status of an organ (Almeida et al., 2007). Since Vechur cattle are less susceptible to mastitis, the *in vitro* experiment assay *TLR*s expression of this breed against mastitis was accessed by challenging the PBMC with LPS. The PBMCs of Vechur cattle were isolated and induced by culturing in presence of LPS in RPM1-1640 media. After culturing, RNA was isolated from the PBMCs and processed for gene expression assay by using RT-qPCR. Derivative melt curve and amplification plot for *TLR2, TLR4*and β-actin genes obtained by RT-qPCR are shown in Figure 1 and 2.

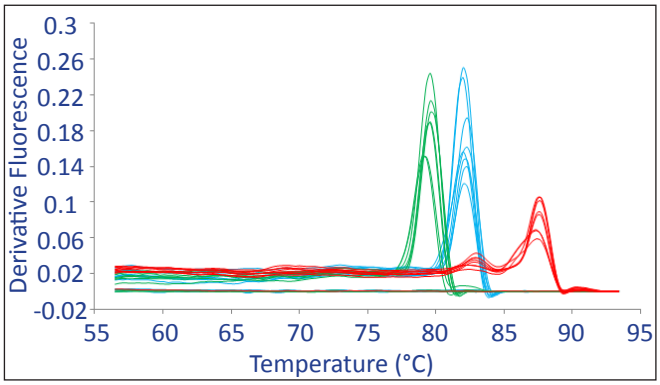

Figure 1: Melt curve plot for *β-actin*, *TLR2* and *TLR4* genes in LPS induction of *in vitro* assay

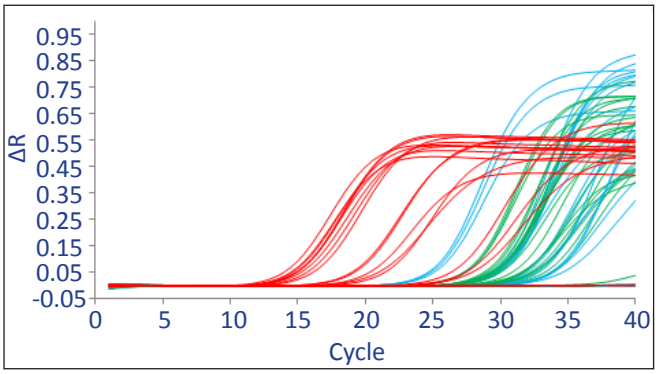

Figure 2: Amplification plot for *β-actin*, *TLR2* and *TLR4* genes in LPS induction of *in vitro*

The mean values of C_q, ΔC_q, $\Delta\Delta C_q$ and relative quantification for *TLR2* gene in Vechur and crossbreed after LPS induction are given in Table 2. The relative expression of *TLR2* was significantly ($p \leq 0.01$) higher in the Vechur cattle (6.90 fold), and also significant ($p \leq 0.05$) in crossbred cattle (1.95 fold) as compared to control (Figure 3). Several studies reported that *TLR2* are capable of mediating LPS responsiveness (Kirschning) et al., 1998; Mastuguchi et al., 2000; Shimazu et al., 1999). Our

Table 2: Relative expression of *TLR2* gene after LPS induction of *in vitro*

Sample	Cq Mean±SE		ΔCq	ΔCq Mean	ΔΔCq	RQ
Sample	*TLR2*	*β-actin*				
Cross breed						
Control	27.11±0.25	14.44±0.27	12.67	12.67±0.25		
Case 1	26.11±0.17	14.06±0.04	12.05		-0.62	1.54
Case 2	26.25±0.05	14.27±0.07	11.98		-0.69	1.62
Case 3	25.45±0.79	14.36±0.27	11.10		-1.57	2.98
				11.71±0.31	-0.96	1.95a^{ns}
Vechur breed						
Control	29.53±0.20	13.16±0.09	16.37	16.37±0.20		
Case 1	26.32±0.06	12.94±0.05	13.38		-2.99	7.94
Case 2	26.57±0.20	12.79±0.39	13.78		-2.59	6.02
Case 3	25.65±0.09	12.06±0.54	13.59		-2.78	6.87
				13.58±0.12	-2.79	6.90^{b**c**}

a: Cross breed vs Control; b: Vechur breed vs Control; c: Cross breed vs Vechur breed

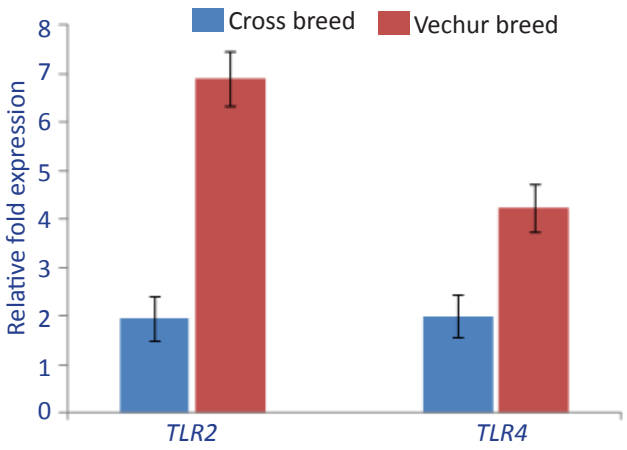

Figure 3: Relative expression of *TLR2* and *TLR4* genes in crossbreed and Vechur cattle

results are in agreement with Ibeagha-Awemuet al., 2008 who found that increased mRNA expression of *TLR2* in mammary glands infected with LPS. Further, Yang et al., 2008 also found increased expression of *TLR2* in mammary epithelial cells collected from diseased quarters of cows challenged with LPS. Medvedev et al., 2000 also described the rapid increase of *TLR2* mRNA in LPS treated mouse macrophage. It has been noted that *TLR2*, can be induced in macrophages in too response bacterial infections and expendable for the initial response (Bannerman et al., 2003). Hoshino et al., 1999 studied on knockout mice confirmed that *TLR4* is critical for LPS signalling. A report by Nomura et al., 2000 reported that *TLR4* mRNA expression was decreased within a few hours of LPS treatment in macrophages, whereas this study could not observe the obvious *TLR4* mRNA decrease after the LPS treatment. In another report Muzio et al., 2000 described that LPS increased *TLR4* mRNA expression in polymorphonuclear leukocytes and monocytes.

host responses against LPS, it may subsidize the stimulated macrophage responses seen at consecutive stages of infection (Mastuguchi et al., 2000). Although LPS is recognized to stimulate various kinase pathways, the activation of pathways does notseem to be crucial for the induction of *TLR2* gene expression. Regulation of *TLR2* expression may be one of the immune regulatory mechanisms commonly involved in host defence against many bacterial strains.

The mean values of C_q, ΔC_q, $\Delta\Delta C_q$, and relative quantification for *TLR4* gene in Vechur and cross breed after LPS induction are given in Table 3. Relative expression of *TLR4* with LPS induction was found to be higher and significant ($p \leq 0.01$) in Vechur cattle than Crossbred cattle (Figure 3). LPS has been reported to up-regulate *TLR4* in bovine mammary epithelial cells (Ibeagha-Awemu et al., 2008). Petzl et al., 2008 also reported increased expression of *TLR4* in mammary tissue after experimental *in vivo* infection with *E. coli.* Similarly, mammary epithelial cells treated with *E. coli* bacteria esulted in up-regulation of *TLR4* (Griesbeck-Zilch et al., 2008) Panigrahi et al., 2014 found that PBMC from crossbred cattle challenged with LPS showed a nearly double and statistically significant increase in mRNA expression of *TLR4* when compared to control group. LPS from *E. coli* and other Gram-negative bacteria are recognized by *TLR4* (Bohan et al., 2019; Poltorak et al., 1998). Inoculation of the mammary glands with bacterial LPS is adequate to activate an inflammatory

Table 3: Relative expression of *TLR4* gene after LPS induction of *in vitro* ass

Sample	Cq Mean ± SE		ΔCq	ΔCq Mean	ΔΔCq	RQ
Sample	*TLR4*	*β-actin*				
Cross breed						
Control	27.11±0.25	14.44±0.27	12.67	12.67±0.25		
Case 1	25.25±0.20	14.06±0.04	11.19		-1.48	2.78
Case 2	26.58±0.25	14.27±0.07	12.31		-0.36	1.28
Case 3	25.88±0.07	14.36±0.27	11.53		-1.14	2.21
					-0.96	$1.99a^{ns}$
Vechur breed						
Control	29.53±0.20	13.16±0.09	16.37	11.68±0.33		
Case 1	27.39±0.42	12.94±0.05	14.45		-1.92	3.79
Case 2	27.23±0.13	12.79±0.39	14.45		-1.92	3.79
Case 3	26.02±0.95	12.06±0.54	13.96	16.37±0.20	-2.41	5.30
				14.29±0.16	-2.08	4.24^{b**c**}

a: Cross breed vs Control; b: Vechur breed vs Control; c: Cross breed vs Vechur breed

Relative expression of *TLR2* and *TLR4* genes was found to be significantly ($p \leq 0.01$) higher in Vechur cattle breed when compared with that of control and crossbreed. Vechur cattle breed are known for its resistance against mastitis, increased expression of *TLR* genes in Vechur cattle during infection

could be related to early regression of infection without precipitating into disease. Bramley et al., 1981 also observed that cows that were challenged with live *E. coli* bacteria showed huge variations in the resistance to mastitis. Joshi and Gokhale, 2006 reported that the genetic makeup of animals leads to the susceptibility to mastitis different from one breed to another. The incidence of mastitis has been found lowest in native breeds of cattle as compared to pure breeds and crossbred cattle. Among the two *TLR*s studied, the relative expression of mRNA in Vechur cattle was relatively higher for *TLR2* (6.90 fold) than *TLR4* (4.24 fold). When Gram-negative bacteria infect the host, LPS was recognized by macrophages through the constitutively expressed *TLR4*. Later, *TLR2* is activated directly by LPS or indirectly through secondary cytokines. Through the newly synthesized *TLR2*, macrophages respond better to LPS or other bacterial components such as lipoproteins that are membranous components of both Gram-positive and Gram-negative bacteria. Hence, higher expression of *TLR2*and *TLR4* might contribute maximum to the innate immune response against the bacteria in Vechur cattle.

4. Conclusion

This study implies that the expression of the two presumptive LPS signalling receptor genes *TLR2* and *TLR4* are differently regulated in Vechur cattle. The expression of *TLR2* was higher when compared to *TLR4*. This may because when gram-negative bacteria infect the host, LPS was first recognized by macrophage, through the constitutively expressed *TLR4*. Latter *TLR2* was activated directly by LPS or indirectly through secondary cytokines. Thus, higher expression of *TLR2* and *TLR4* might contribute maximum to the innate immune response against the bacteria in Vechur cattle.

5. Acknowledgment

The authors acknowledge College of Veterinary and Animal Science for providing the laboratory facilities for the successful completion of this work. The first author acknowledging the Department of Science and Technology for providing INSPIRE Fellowship (IF131025) for the Ph.D program.

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