Research

Differential Expression of Pattern Recognition Receptor (PRR) Genes in Zebu (*Bos indicus*) and Crossbred (*Bos taurus* x *Bos indicus*) Cattle in Response to *Brucella abortus* S19 Antigen

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ABSTRACT

Innate immunity is critical for the initial containment of the pathogen in the host; however, the strength may deviate in different host types. Here, we demonstrated the relative expression of pattern recognition receptor (PRR) molecules-Toll-like receptor (TLR) 4 and NOD-like receptor (NLR) 1 of immune cells, involved in the initial recognition of pathogens, during innate immunity in *Bos indicus* (zebu) and *Bos taurus*× *Bos indicus* (crossbred) genetic groups. Real-time expressions of *TLR4* and *NLR1* genes were evaluated in six female young calves of both groups before (0d) and after 7th, 14th, and 28th day (d) immunization with attenuated *Brucella abortus* S19 strain. The receptor genes, in both of the groups, showed downregulation during the initial stage (at 7d) of immunization, followed by upregulation at a later stage. The expression of the *TLR4*, a specific PRR to recognize gram-negative bacteria including *B. abortus*, was significantly ($p<0.01$) higher in crossbred than that of zebu at 14d and 28d. However, the expression of the *NLR1*, a generalized PRR, was significantly ($p<0.05$) higher initially (14d) in crossbred, but later (28d) in zebu. The differential expression of PRR molecules observed in both cattle groups indicated their diverse pattern and strength of containment of the *Brucella* organism at an initial initial stage.

Keywords: *Brucella abortus* S19, Brucellosis, Cattle, PRR molecules, Immunomodulation

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INTRODUCTION

A class of antigen-binding receptor molecules located on immune cells plays a pivotal role in initiating long-term immune response, through modulating at the innate and acquired levels. Macrophages and dendritic cells, which recognize the intracellular pathogens, including *Brucella*, destroy them along with the infected host cells and further modulate the immune system with the help of such receptors (Thakur *et al.,* 2019). The receptors like Toll-like receptor (TLR) and Nod-like receptor (NLR) recognize the pathogen-associated molecular patterns (PAMPs) of the bacteria and initiate phagocytic activity and other signaling pathways. TLR4 specifically recognizes the gram-negative bacteria originated lipopolysaccharide (LPS), whereas NLR1 is non-specific receptor, recognizes a wider spectrum of PAMPs of pathogens (Mogensen, 2009). Such initial recognition of the pathogen is a critical step for early containment of the pathogen as well as initiating the adaptive immune response.

Brucellosis, caused by intracellular *Brucella* species is a devastating zoonotic disease. Cell-mediated immune response is primarily responsible for the clearance of intracellular *Brucella*. The organism remains inside and replicates in phagocytic cells of the reticulo-endothelial system as well as in non-phagocytic cells like trophoblasts. Macrophages, dendritic cells have foremost roles in recognizing the invading *Brucella* and destroying them along with the infected host cells through phagocytic or cytotoxic activity (Baldwin and Goenka, 2006; Olsen *et al.,*, 2010). *B. abortus* is prompt in the production of pro-inflammatory cytokines such as interleukin by different kinds of cell types (Zhan *et al.,* 1993; Zaitseva *et al.,* 1996; Huang *et al.,* 2003; Cha *et al.,* 2010). These actions of T cells can kill the invading *Brucella* organism both directly and indirectly (Zhan and Cheers, 1995). TLRs recognize the PAMPS on the pathogen, and the immune response relies mostly relied upon this process of pathogen recognition. After recognition, these receptors trigger a signaling pathway, leading to the secretion of proinflammatory cytokines, chemokines, and interferons as a cellular response (Kawai and Akira 2010). TLR2 and TLR4 are involved in recognizing the PAMPs on *Brucella* antigen during processing (Giambartolomei *et al.,* 2004, Pasquevich *et al.,* 2010).

Evidence suggests that the immune response against *Brucella* sp. seems to vary in view of the differential genetic makeup in the cattle population. In bovines, inter-breed and interspecific differences in susceptibility against brucellosis have also been

reported (Kamboh *et al.,* 2007; Ali *et al.,* 2013). Although maternal antibodies pass vertically to calves through colostrum, however, these antibodies may not be enough to protect the animal against brucellosis (Rhyan *et al.,* 2009). Therefore, immunization seems to be the most effective measure to reduce the prevalence of brucellosis. *B. abortus* Strain 19, a live attenuated vaccine, is widely used for control of bovine brucellosis with high immunogenicity and antigenicity (Miranda, *et al.,* 2015). India, with a cattle population of 190 million heads, is the second highest in the world.

Crossbreds (*Bos taurus*× *Bos indicus*) mostly have 50% or more blood of exotic cattle (Holstein Frisian or HF and Jersey), with 19% of the total cattle population contributing more than half of the total cow milk in the country. The milk productivity is almost 3 times higher in crossbred cattle than in indigenous cattle of the country. Occurrence of *Brucella* infection has been reported with varying degrees in crossbreds and the indigenous cattle population. Some of the regions in India have reported 8.85% percent of seroprevalence of *Brucella* organism in the bovine population (Upadhyay, 2007). Indigenous cattle with 4.4%, exotic with 6% and crossbred with the highest seroprevalence 7.8% (Ali *et al.,* 2013). Indigenous cattle with 28.40% and crossbred with 52.30% (Patel *et al.,* 2014). For their prevention, immunization with the S19 strain is the most preferred method with these areas. However, very few studies have been conducted to extrapolate the immunogenetic pathways after *Brucella* S19 immunization in *Bos indicus* (Zebu) and *Bos taurus* × *Bos indicus* (crossbreds). Hypothesizing the genetic variation in immune response in both cattle types with different genetic composition, the current study was intended to extrapolate differential expression of some specific genes coding for cytokines and antigen receptor molecules against *B. abortus* S19 antigen in *Bos indicus* and *Bos taurus* × *Bos indicus* cattle.

MATERIALS AND METHODS

Experimental animals and design

The experiment was conducted in a brucellosis-free herd located at ICAR-National Dairy Research Institute (NDRI), Karnal, India. Immunization with Brucella S19 antigen is practised routinely in young female calves of 4 to 8 months of age in the herd every year. For our experiment, six female calves aged between 4 to 6 months were randomly selected for 3 groups: crossbred (*Bos taurus* × *Bos indicus*), Karan Fries, and Sahiwal (*Bos indicus*). The animals were confirmed to be brucellosis-negative for brucellosis by doing serological testing with RBPT. Crossbred Karan Fries cattle population is a result of the planned crossbreeding of indigenous zebu Tharparkar (*Bos indicus*) and exotic Holstein Frisian (*Bos taurus*) breeds that were stabilized at 50 percent exotic inheritance through *inter se* mating for >10 generations. The Sahiwal is a native zebu cattle breed of the Indian subcontinent, well known for high milk productivity in tropical conditions along with tick resistance, heat tolerance and less disease occurrence. Both groups of calves were reared in separate but adjacent sheds under an intensive management system supported with *ad-lib* feed resources and supplements. The experiment was conducted with the approval of the Institute Animal Ethics Committee of the National Dairy Research Institute, Karnal, India.

Brucella S19 Immunization

The calves were vaccinated with Live IP Vet, Freeze-dried *Brucella abortus* Strain 19 vaccine (MSD Animal Health, Intervet India Pvt. Ltd., India). The vaccine was administered subcutaneously in 2ml containing a dose of 40×10^9 viable organisms. All calves were vaccinated on a single day in the month of November (ambient temp 28º C) and were kept under observation during experimentation. Blood samples were collected from all the calves just before immunization (0d) and (7d), (14d), and (28d) days of immunization. About 10 ml and 2ml of blood were collected aseptically from the external jugular vein in two different sterile EDTA EDTA-coated and plain serum vacutainer tubes for isolation of PBMC from whole blood and estimation of immunoglobulin from serum, respectively. Blood samples were collected with the least time difference (with 15-20 minutes) from both of the groups, and further, the collected samples were transported to the laboratory within an hour so that any degradation of RNA could be avoided. Serum was harvested from all blood samples collected separately without an anticoagulant and stored at -20°C till further use.

RNA extraction and cDNA synthesis

Peripheral blood mononuclear cells (PBMCs) were harvested from blood samples using a density gradient centrifuge following standard protocol. TRIzol reagent (Invitrogen, Corp., CA) was used to homogenize the PBMC. RNeasy Mini kit columns (Qiagen, Germany) was used for purification of RNA. Further, RNA was quantified by Nanodrop spectrophotometric analysis. OD ratio of the samples at A260/280 by the Nanodrop spectrophotometer ranged from 1.8-2.0, indicating good quality of the collected RNA. The integrity of RNA samples was further confirmed by using agarose gel electrophoresis. From the extracted RNA, complementary DNA (cDNA) was synthesized using Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas, USA) as per manufacturer instructions. The semi-quantitative PCR was performed with *RPS15* gene in all samples to cross-check the cDNA integrity.

RPS15 is suitable as a reference gene for accurate normalization of PBMC transcripts (Kishore *et al.,* 2013). The primer sequences of house-keeping gene *RPS15* and target genes - *TLR4*) and *NLR1* were either designed through Primer Express 3.0 software (Applied Biosystem) or taken from literature (Im *et al.,* 2016).

Real-Time PCR quantification

The expression of target genes in PBMCs of indigenous and Karan Fries calves was measured by real-time quantitative PCR in Roche Light Cycler® 480 II machine using Roche SYBR Green. The primers used are given in Table 1. Each reaction in a 96 well transparent plate (Thermo, USA) comprised of 4 µl diluted cDNA combined with 6 µl of mixture composed of 5 µl Roche SYBR Green master mix (2x), 0.3 µl each of 10 µM forward and reverse primers, and 0.4 µl DNase/ RNase free water. For each gene, samples were run in duplicate form along with six-point relative standard curve plus the non-template control. The reactions were performed with amplification conditions: 10 min at 95°C, 40 cycles of 15s at 95°C (denaturation) and 1 min at 60°C (annealing + extension). A dissociation protocol with an incremental temperature of 95°C for 15 s plus 65°C for 15 s was used for specificity of the qPCR reaction.

Table 1: Description of primers for qPCR of PRR genes in cattle

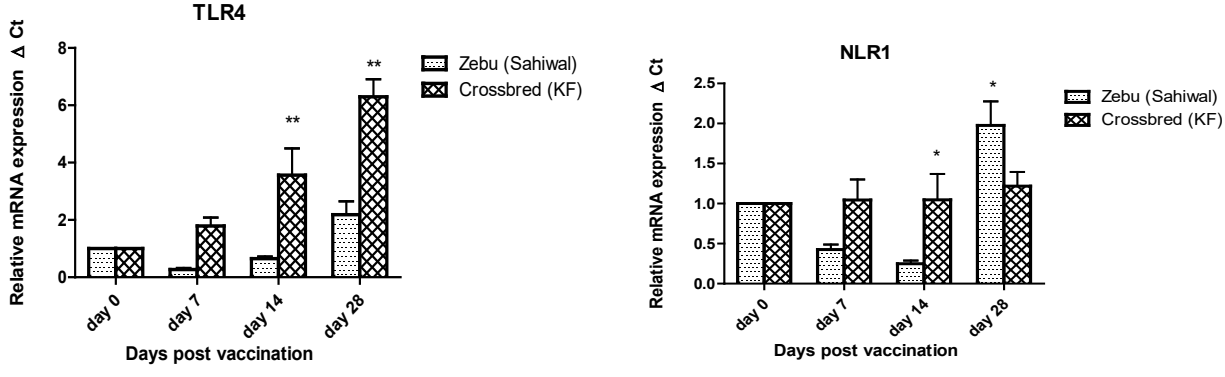
Gene	Sequence (5'- 3')	Annealing Temp. (°C)	Product size (bp)	References
RPS 15	CAGCTTATGAGCAAGGTCGT GCTCATCAGCAGATAGCGCTT	60	150	Kishore *et al*., 2014
TLR 4	CCCTTTCAGCTCTGCCTTCACTAC AGGTCTGGGCAATCTCATACTCG	60	143	Designed under this study
NLR 1	CCAGGGGCTCAGAACTAACA CCACCTCAAGCTCTGGTG	60	118	Designed under this study

Relative expression of target genes

The analysis of mRNA expression data for all the genes across different samples was based on cycle threshold (Ct) values. The Ct values of each of the target genes were deducted from the arithmetic mean of Ct values of *RPS15* to calculate ΔCt. To compare the relative expression level of different genes, the ΔΔCt values were analyzed using a two-way ANOVA test using GraphPad PRISM version 5.0 statistical software (La Jolla, CA, USA). Within the animal, ΔCt values were analyzed by one-way ANOVA by Tukey's multiple comparison tests.

RESULTS

In this paper, we studied the immune modulation of receptor molecules-TLR and NLR involved in the recognition of molecular patterns of live attenuated *Brucella* S19 strain during the initial days of immunization in Sahiwal (zebu) and KF (crossbred) female calves maintained in the subtropical region of India. The study revealed a differential expression pattern in both the groups (Fig.1). The receptor genes showed initial decline (at 7d) in expression after immunization, and further upregulation in both groups at 28d. The expression of *TLR4* in KF calves continuously increased after immunization-1.8-, 3.6- and 6.3-times at 7d, 14d, and 28d, respectively, in comparison to the basal level (0d); however, the increase (2.2 times) from the basal level was observed on 28d only in Sahiwal. Between the genetic group comparison, the *TLR4* expressions were higher in crossbred than that of Sahiwal at all days of the immunization however, significantly ($p<0.01$) higher at 14d and 28d. Expression of *TLR4* was five times higher (3.6vs 0.7) in KF than that of Sahiwal on 14d and about three times higher (6.3 vs. 2.2) on 28d.



**Significance level ($p<0.01$), compared between two groups.

Fig. 1: *Relative mRNA expression (ΔCt) of immune response genes in different days after Brucella S19 vaccination in Zebu (Sahiwal) and crossbred (Karan fries) calves a) TLR4; b) NLR1.*

In contrast, the expression of *NLR1*, another recognition molecule, remained almost static post immunization in crosses, whereas it modulated erratically in Sahiwal calves initially decreased on 7d (0.4 fold change) and 14d (0.2 fold change), followed by 2 fold increase on 28d compared to the expression level before immunization. The expression of NLR1 in zebu calves was increased about 10 times between 14d and 28d; in comparison to a subtle increase in crossbreds. The expression level of NLR1on 28d in zebu calves was found significantly ($p<0.05$) higher than that in KF.

DISCUSSION

An important step during the initiation of the immune response is the process of pathogen recognition by pathogen recognition receptors (PRRs) like TLRs and NLRs, and subsequently triggering multiple proinflammatory signaling pathways, leading to further secretion of proinflammatory cytokines as a cellular response (Kawai and Akira, 2010; Pasquevich *et al.,* 2010). During Brucellosis, initial immunity seems to be important in restricting the intra cellular *Brucella* organism; however, only a minor protective effect has been observed in bovines (Bellaire *et al.,* 2005; Baldwin and Goenka 2006). Pathogen receptor molecules, mainly TLR and NLR become more important as a key step for further signaling for the progression of immunity against the most intracellular pathogen bacteria. The TLRs, specifically *TLR4*, are also required for clearance of the *Brucella* from different organs in the host (Pei *et al.,* 2012).

Our results revealed that the *TLR4* was found to be upmodulated from the beginning in KF, however, later in Sahiwal calves, after initial down regulation. The *Brucella* organism has also been seen to downregulate the *TLR4* initially followed by upregulation, depending on the doses, in other studies in cattle (Im *et al.,* 2016), in mice (Nomura *et al.,* 2000). Initial modulation of *TLR4* in calves might be most critical wherein, the crossbreds might have an advantage over the zebu during initial clearance of the *Brucella* from the host. Significant differences in the *TLR4* expression in zebu and crossbreds at later days also indicated the same. Higher interaction of *TLR4* in KF also explains the higher expression of proinflammatory cytokines (IL6, IFNγ) in KF. In our earlier study on expression of cytokine genes (*TNFα, IFN-γ, IL-6* and *IL-10*) in the same genetic groups also revealed initial downregulation followed by upregulation in both the groups, similar to antigen receptor molecules in response to *Brucella* S19 immunization (Ravi Kumar *et al.,* 2019).

Another PRR, the *NLR1*, does not seem to have an important role in zebu cattle, which had some drastic change in expression after immunization. On the other side, Karan Fries showed almost constant expression during post-immunization days. Although the NLR-based signaling pathway for host defense is predominant, mainly against extracellular and some intracellular bacteria (Kobayashi *et al.,* 2005, Keestra *et al.,* 2011, Kim *et al.,* 2004, Ferwerda *et al.,* 2005). Since, the NLRs have been least studied for their role in intracellular infections, the significant modulation, specifically about 2 times higher expression on 28d than the basal level in Sahiwal calves, their role specific to *Brucella* needs to be studied in cattle breeds further.

Increased expression of PRR molecules may be indicative of intiating the antibody-based adaptive immunity against the pathogen. In our earlier report (Ravikumar *et al.,* 2019), antigen-specific total IgG was determined by Indirect ELISA, which revealed 3 to 3.5 times higher values after immunization, confirming the initiation of an immune response against *Brucella* in both of the genetic groups. From our results it is clear that both of the genetic groups showed an increasing trend of expression after immunization; indicating the S19 vaccine would also induce an immune response at the initial stage of immunization in cattle.

CONCLUSION

This study shows the differential real time expression of important receptor genes (*TLR4, NLR1*) in zebu and crossbred cattle groups after vaccination with the attenuated *B. abortus* S19 strain. All three receptor genes showed initial decline in expression, just after immunization, followed by significant increase at a later stage in both of the groups. Importantly, the expression of the *TLR4* was significantly ($p<0.01$) higher in crossbred than that of Sahiwal at 14d and 28d. The *NLR1* revealed some erratic pattern in zebu calves, with about 10 times increased expression between 14d and 28d; in comparison to nearly static expression in crossbreds. The study indicated that increased expression of PRR molecules after *B. abortus* S19 immunization may be critical for initial containment of the pathogen as well as in triggering the long-term immunity in both cattle genetic groups, although in slightly different ways.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

COMPETING INTERESTS

The authors have declared that no competing interests exist.

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