Major histocompatibility complex (DRB3) gene expression pattern indicates differences in *Brucella abortus* S19 vaccine induced immune response in Karan Fries and Sahiwal cattle

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**ABSTRACT**

*Brucella abortus* S19 strain vaccination is most effectively used as a tool to control the brucellosis in cattle. To understand the genetic basis of differences in immune responsiveness after immunization in cattle of different genotypes, we assessed the expression of MHC-DRB3 antigen receptor molecule in six each female calves of Karan Fries crossbreds (KF, *Bos indicus x Bos taurus*) and Sahiwal (*Bos indicus*) vaccinated with *Brucella* S19. Serum and peripheral blood mononuclear cells (PBMC) were isolated from blood collected on 0 (before vaccination) and 7, 14 and 28th day of vaccination. Antigenic response was assessed for these days in both the groups using Rose Bengal Plate form Test (RBPT). At 0d, the calves of both groups showed no antigen agglutination, confirming the calves free from the infection. The serum of 7d onward started showing the agglutination with more strong response in later stages specifically in KF, indicating increased immune response against *Brucella*. Therefore, RBPT can be used as earliest screening (7d onward) for *Brucella* antigenic reactivity in both cattle groups. The expression of DRB3 gene started with slight upregulation after vaccination. In general, however, without any significant differences between two different genetic groups up to 14d. The significant (p<0.01) higher expression (8 times) of DRB3 was observed in KF than Sahiwal at 28d. The study indicated that antigenic reactivity and MHC-DRB3 expression elicited by *Brucella* S19 vaccination was more prominent in KF during initial days, which may provide an extra advantage to the host for antigen binding, thereby better immune protection at later stage.

**Key words**: *Brucella abortus* S19, Brucellosis, Cattle, Gene expression, MHC-DRB3.

**INTRODUCTION**

Brucellosis has posed a serious threat to the animal production system through large economic losses and health implications, worldwide. It has also put the negative impact on public health as World Health Organization (WHO) classified it as one of the leading ‘neglected zoonotic diseases’ (Corbel, 1997; WHO, 2016). Brucellosis in different species, based on their primary host is caused by *Brucella* species: *B. melitensis* (sheep and goats), *B. abortus* (cattle), *B. suis* (swine), *B. ovis* (sheep), *B. canis* (dogs) and *B. neotamae* (desert wood rats) (Olsen et al., 2004). In cattle, natural infection of *B. abortus* occurs primarily through penetration of mucosal membrane of the alimentary tract (Adams, 2002), which further transported, either free or within phagocytic cells to the regional lymph nodes of the host. Placentitis, abortion and temporary infertility are the principal clinical manifestations of brucellosis in cattle.

The control of brucellosis is done largely by vaccination of young animals as culling seems to be extremely costly and not realistic under high disease prevalence conditions (Moreno, 2002; Office International des Épizooties, 2013). *B. abortus* S19 is a live attenuated vaccine with high immunogenicity and antigenicity and considered to be the first vaccine widely used for control of bovine brucellosis (Manthei, 1968, Miranda, et al., 2015). However, the strain does not seem to protect all cattle, pointing out the genetic basis of adequate immune responsiveness in bovines (Davies, 1980; Deyoe, 1979; Garcia, 1980). Since, differential immune responses, both at innate and acquired levels contribute significantly during controlling the brucellosis (Blasco et al., 1993; Verger et al., 1995; Moriyón et al., 2004; Godfroid et al., 2011), the expression of proinflammatory cytokines and pathogen’s antigen receptor molecules seems to play important role in initial containment of *Brucella* organism inside the host.

Major histocompatibility complex (MHC) class II molecules are one of the major antigen binding molecules, which binds with the *Brucella* antigens and present them to T helper cells, thus influencing downstream adaptive immune process during disease progression (Dietz, 1997). *Brucella* also found, although not as a general principle, to inhibit the

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expression of MHC II molecules to evade from immune response; interfering the antigen processing in chronic condition (Murphy et al., 2002, Barrionuevo et al., 2008). A few studies investigated the role of MHC class II molecules on account of variation in responsiveness to Brucellosis vaccination in cattle, although no study have been conducted in Indian cattle, till now. This work reports the comparative study on MHC class II-DRB3 expression pattern in Sahiwal and Karan Fries cattle after Brucella abortus S19 vaccine to extrapolate the genetic basis of differences in immune responsiveness after immunization in these cattle of different genetic background.

**MATERIALS AND METHODS**

**Experimental animals:** Six healthy 4-6 months old female calves each of Karan Fries (KF, Bos indicus x Bos taurus) crossbred and Sahiwal cattle were marked randomly for the experiment at ICAR-National Dairy Research Institute (ICAR-NDRI), Karnal, India. The experiment was conducted with the approval of Institute Animal Ethics Committee of National Dairy Research Institute, Karnal, India. Karan Fries (KF) cattle are a crossbred synthetic population with 50-70% of Holstein Friesian inheritance, originated through crossbreeding of Tharparkar (Bos indicus) and Holstein Frisian (Bos taurus). The calves initially confirmed for free from brucellosis by Rose Bengal Plateform test (RBPT), were isolated separately and vaccinated with Live IP Vet, Freeze dried Brucella abortus Strain 19 vaccine (MSD Animal Health, Intervet India Pvt. Ltd.) on a single day of November month (at onset of winter). The vaccine was administered subcutaneously of about 2ml containing a dose of 40 x10⁶ viable organism. Blood samples for serum and peripheral blood mononuclear cell (PBMC) isolation were collected in separate vacutainer tubes just before vaccination (0 day) and 7th day, 14th day, 28th day after vaccination for each calf.

**Serum isolation and Rose Bengal Plateform test:** Serum was harvested from all blood samples collected separately without anticoagulant and stored at -20°C till further use. Serum samples collected from the calves were subjected for screening of brucellosis using agglutination based Rose Bengal Plateform test (RBPT), to confirm the reactivity of the antigen before and after vaccination. The reaction mixture consisted equal amount of serum and antigen. Total volume (25-30µl) of serum and Brucella antigen were placed side by side on a plate and further mixed thoroughly and rapidly by light shaking of plate for about 4 minutes. Results were assessed through agglutination of the antigen.

**Peripheral blood mononuclear cell isolation and RNA extraction:** After blood collection the samples were immediately processed to prevent RNA degradation. Blood were diluted with Phosphate buffer saline (PBS) in 1:1 ratio. The peripheral blood mononuclear cell (PBMCs) were isolated by standard protocol using Histopaque-1077(Sigma) density gradient purification. Briefly, 5 ml blood collected from each calf was overlaid onto 3 ml of Histopaque in a 15 ml conical tube and further centrifuged at 400g for 30 minutes. PBMC band at the interphase of plasma and Histopaque harvested and washed twice with PBS. After purification of PBMCs, RNA extraction was carried out using TRIzol reagent (Invitrogen, Corp., CA) as per manufacturer’s protocol. RNA was further purified using RNeasy Mini kit (Qiagen, Germany) followed by on-column digestion with RNase-free DNase (Qiagen, Germany). Quantification of RNA was done using Nanodrop ND-1000 spectrophotometer (Nano Thermo Fisher Scientific). The quality of RNA was checked using 1.5% agarose gel. The RNA used in the present study was found to be of good quality and the agarose gel electrophoresis revealed three distinct bands (5S, 18S and 28S) (Fig.1).

**cDNA amplification and Real time expression:** From the purified RNA, cDNA was synthesized using Revert Aid™ First strand cDNA Synthesis Kit (Fermentas, USA) following manufacturer’s protocol. Total 1.0 µg of RNA was reverse transcribed by adding 1.0 µl Oligo(dT)₁₅ primers (0.5 µg/µl) and made the total volume of 12 µl by adding DEPC-treated water. Further, the mixture was incubated at 65 °C for 5 min and subsequently snaps chilled on ice. A total of 8.0 µl of master mix consisting of 4.0 µl 5X First-Strand buffer, 1.0 µl M-MuLV Reverse Transcriptase (200 U/µl), 1.0 µl RiboLock™RNAase inhibitor (20U/µl) and

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**Table 1: Primers used for Real time qPCR.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (5'-3')</th>
<th>Annealing temp. (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
</table>
| RPS15      | Forward: CAGCTTATGACACAGGTCTGT  
             Reverse: GCTCATCAGCAGATACCCGTT | 60                  | 180               |
| MHC-DRB3   | Forward: TGGAAAGGCCATCCATCCATCT  
             Reverse: CCCACTGTGATGTGGCCAG | 60                  | 169               |
2.0 µl 10 mM dNTP mix was prepared for PCR. The PCR reaction was performed with the programme: 42 °C for 1 hr, 70 °C for 10 min and hold at 4 °C for 5 min.

Primer sequence for target gene MHC-DRB3 was designed through primer express 3.0 Software (Applied Biosystem)(Table 1). The DRB3 gene was amplified from cDNA samples using semi quantitative PCR technique in Sahiwal and Karan Fries cattle. The relative gene amplification of DRB3 gene was performed in Roche Light Cycler® 480 Light CyclerII. Each reaction (10.0 µl) in a 96 well plate consisted of 2 µl diluted cDNA and 8 µl of 2X Roche SYBR Green master mix (USB, Affymatrix), 0.3 µl each of 10 mM forward and reverse primers and 2.4 µl nuclease free water. The reaction conditions were: 95 °C for 10 min followed by 40 cycles of 15 sec at 95 °C (denaturation) and 1 min at 60 °C (annealing and extension). Each reaction was performed in duplicate along with the non-template control. RPS15 gene was included as internal control gene to normalize the expression data of target candidate genes (Kishor et al., 2013). The melting curve was produced after completion of the qPCR to assess the specificity of the amplified products.

Data analysis: Average Ct values of internal control gene RPS15 was used to normalize the target gene expression data. The analysis of mRNA expression for different samples was based on cycle threshold (Ct) values. The Ct values of MHC-DRB3 mRNA were normalized with the mean Ct values of the RPS15 to calculate ΔCt. For comparative analysis, ΔΔCt values of each breed/population used to calculate the fold change in expression as described by Livak and Schmittgen (2001). Student’s T-test was employed to analyze significance of expression difference of genes across breeds. To compare the relative expression level of different genes, the ΔΔCt values were analyzed using 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 test by Tukey’s multiple comparison tests. The p value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSIONS

Newman and coworkers (1996) reported that Brucella abortus S19 vaccine produces a large variation of immune response for different hosts. In bovines, inter-breed and inter-species differences in susceptibility against brucellosis were also reported (Kamboh et al., 2007; Ali et al., 2013). Cows with different genetic background have shown different phagocytosis activity to control of Brucella (Price et al., 1990). Therefore, to elucidate the differential immune response against Brucella in two cattle groups of different genetic background- Sahiwal and Karan Fries, we studied the antigenic reactivity as well as relative gene expression of MHC-DRB3 molecule in these two groups.

First, serum samples collected at different days (0d, 7d, 14d and 28d) of all the calves under experiment were subjected to the RBPT for confirming the reactivity of Brucella antigen as an immune responsiveness against antigen (Diaz et al., 2011) (Fig. 2). Before vaccination (0d), the results of agglutination by RBPT showed no agglutination between the serum and Brucella antigen under the test, indicating the calves free from brucellosis before vaccination. However, one calf showed false positive at 0d which was further confirmed by indirect ELISA test. In most of the calves, the serum samples 7 days after vaccination showed agglutination of antigens, indicating positive results. In some animals, Brucella antigen agglutination based positive results could be obtained only after 14d. On the 14th and 28th day, most of the crossbred calves showed comparatively stronger antigen agglutination, reflecting higher immunoglobulin concentration there by higher immune response after the vaccination. Generally antibody titers after vaccination may continue for a period in a minor proportion of vaccinated calves. Since, the Rose Bengal Platform Test is done with buffered Brucella antigen based on a rapid agglutination test, it sometimes gives false positive result in form of antigen agglutination, causing misinterpretation of the results (Irmak et al., 2004, Corbel et al., 2006). Therefore, the RBPT should be validated with indirect Enzyme Linked Immunosorbent Assay (ELISA), recommended by World Health Organization (Corbel, 2006). Not with standing, it can be concluded that RBPT can be used for earliest screening for antigenic reactivity by 7th day in both indigenous and crossbred cattle, however, it should be validated with more sensitive tests like indirect ELISA.

The relative expression of the MHC-DRB3 and RPS15 genes was assessed by real time PCR from the melting curve and amplification plot (Fig. 3). It was ensured that each primer pair had a good accuracy by observing the presence of a single unique peak of melting curve at the end of the reaction. Like-wise amplification plot showing
Table 2: Relative mRNA levels (Mean ± SE) of DRB3 gene.

<table>
<thead>
<tr>
<th>Days</th>
<th>Karan Fries</th>
<th>Sahiwal</th>
<th>Significance Level between Breed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>0.7483 ± 0.1980</td>
<td>1.231 ± 0.5732</td>
<td>NS</td>
</tr>
<tr>
<td>Day 14</td>
<td>1.911 ± 0.3651</td>
<td>1.612 ± 0.8484</td>
<td>NS</td>
</tr>
<tr>
<td>Day 28</td>
<td>7.896** ± 2.052</td>
<td>0.9617 ± 0.2918</td>
<td>(p&lt;0.01)**</td>
</tr>
</tbody>
</table>

After standardizing the mRNA expression data, DRB3 in Karan Fries calves, were found to be slightly down regulated on 7th day but further up-regulation on 14th day. In both Sahiwal and Karan Fries cattle, there was no significant difference between the days (Table 2). Significant (p<0.01) up-regulation in KF was observed on 28th day with a mean value (7.896 ± 2.052) when compared to that of expression at 0d (before vaccination), whereas, the expression level was also 8 times higher than Sahiwal calves at same time point.

It have been reported that after S19 vaccination immune response varies in large amount between indigenous and jersey crossbred (Newman et al., 1996; Verma et al., 2017), our study also indicated a large difference in agglutination as well as DRB3 expression levels between crossbreds and indicus cattle. The increase in expression of DRB3 gene in KF calves was also corroborating with results of higher agglutination compared to the Sahiwal cattle. Almost no change in gene expression of DRB3 gene in Sahiwal cattle could be an indication that the indigenous population might be less immune responsive or sensitive to the Brucella, however, other non MHC genes should also be studied in larger number of calves to make for validation of this possibility.

Brucella also found to evade immune response through inhibiting the expression of MHC II molecules and interfering with antigen processing (Barrionuevo et al., 2008). The course of evasion becomes important for long term stay of the organism inside the host cells. However, decreased MHC expression does not always associate with Brucella infection (Murphy et al., 2002). The DRB3 is not seem to be much induced initially by Brucella antigen; however upregulation in later stage indicates Brucella organism may not succeed in down regulating the MHC class II molecule for immune evasion, at least in initial stage. Moreover, higher expression of MHC-DRB3 at later stage also seems advantageous in binding of antigens for further processing and immune regulation.

CONCLUSION

We have shown that vaccine induced immune response in Sahiwal and Karan Fries calves, however differed significantly. In crossbred KF cattle immune response elicited by Brucella S19 was higher than taurine Sahiwal, which showed lower antigenic reactivity as well as almost static expression of BoLA-DRB3 gene. More prominent immune response in KF during first month of vaccination may provide an extra advantage to the host for antigen binding, thereby better immune protection. On other side, Sahiwal calves were found less immune responsive to the Brucella, which could be an indication of their lower sensitive or higher resistance to Brucella. To validate, both views, however, more extensive studies on other immune response genes for longer duration are required.

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