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RESEARCH ARTICLE

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Differential cytokine response of *Escherichia coli* lipopolysaccharide stimulated peripheral blood mononuclear cells in crossbred cattle, Tharparkar cattle and Murrah buffalo - An *in vitro* study

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Abstract

Mastitis is a complex disease responsible for huge economic losses to the dairy sector. The causal organisms include a wide variety of micro-organisms including several species of bacteria. *Escherichia coli* has been identified as one of the most common gram-negative bacteria causing clinical mastitis in cattle. The immune system, of different species and/or breeds, tries to combat these pathogens in an inconsistent manner with differential mode and intensity of immune response, eventually producing contradicting outcomes of this disease. Several reports suggest the existence of variability among different animal breeds/species, resulting in a dissimilar outcome of this disease among them. In order to evaluate the variation among different breeds/species, the present study was undertaken to examine the stimulant effect of *E. coli* lipopolysaccharide (LPS) on peripheral blood mononuclear cells (PBMCs). The PBMCs were harvested from blood samples of crossbred cattle, Tharparkar cattle and Murrah buffaloes. After 6 h of *in vitro* stimulation, qRT-PCR was employed to measure the relative mRNA expression levels of *CCL5, IL-1β, IL-12β, IFN-γ* and *IL-10* genes in stimulated and unstimulated PBMCs. The selected genes revealed significant differences in the pattern of innate immune response among crossbred cattle, Tharparkar cattle and Murrah buffaloes. The results clearly indicate the presence of variation in the outcome of immune response even when the immunocytes were stimulated with the same dose of the antigen.

Additional keywords: antigens; breeds; expression; immunity; mastitis.

Abbreviations used: CMT (California Mastitis Test); LPS (Lipopolysaccharide); PAMP (Pathogen associated Molecular Pattern); PBMC (Peripheral Blood Mononuclear Cell); PCR (Polymerase Chain Reaction); PRR (Pattern Recognition Receptor); SCC (Somatic Cell Count).

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Introduction

Escalating mastitis cases courtesy mainly the mismanagement and emergence of antibiotic-resistant bacterial strains are causing great economic breakdown across the world (Aslantaş & Demir, 2016). Mastitis, an inflammatory disease of the mammary gland, is caused by a wide variety of pathogenic microorganisms. Among them, *Staphylococcus aureus* and *Escherichia coli* are the most common (Bannerman, 2009). With multiple aetiological agents, mastitis problems remain vague via strategic use of the effective vaccine (Detilleux *et al.*, 1994). Apart from managemental care, selection of disease resistant animals into the herds is a promising approach for resolving mastitis. Genetic selection of cattle populations in Scandinavian countries has been performed on the basis of udder health and recorded clinical mastitis case (Heringstad *et al.*, 2007). The

inclusion for sire evaluation in these breeding programmes has resulted in a lower trend of clinical mastitis cases in these countries (Sender *et al*., 2013).

The innate immune system of an animal tries to combat a broad variety of pathogens and is based on a limited repertoire of germline-encoded receptors called pattern recognition receptors (PRRs) of immunocytes. PRRs of immunocytes sense specific Pathogen Associated Molecular Patterns (PAMPs) of pathogens and lead to the production of cytokines which in turn bridge innate and adaptive immune responses (Mogensen, 2009). Lipopolysaccharide (LPS), the outer component of gram-negative bacteria such as *E. coli*, binds with LPS binding protein that in turn binds with CD14/TLR4/MD2 receptor complex and eventually induces cytokine release from various kinds of cells (Gerold *et al*., 2007; Lu *et al*., 2008). Through its involvement in different pathways, LPS has been reported to induce a pro-inflammatory response in different mastitis-models (Xiao *et al*., 2015). White blood cells of the vascular system especially lymphocytes, monocytes and macrophages effectively regulate the inflammation process by acting as the first line of immune defence against invading pathogens (Mogensen, 2009). These cells have the ability to produce inflammatory mediators like the cytokines and chemokines that act by attracting other immune cells to the site of inflammation. Lymphocytes that invade the mammary gland during infection are present in its secretions and participate in an active role by providing protection against important mastitis causing organisms (Park *et al*., 2004).

Animal hosts, with diverse genetic makeup, encounter numerous pathogens with varying virulence. The genetic make-up, physiological state of the host and the traits of the etiological agents govern about the disease tolerance and/or susceptibility in the host (Zanella, 2016). Disease resistance is a threshold trait which follows polygenic inheritance but varies phenotypically in a discontinuous manner *i.e*., either normal or diseased (Falconer & Mackay, 1996). Breed-wise and species-wise differences in susceptibility pattern towards mastitis have been well documented (Rupp & Boichard, 2003). Buffaloes in comparison to crossbred cattle and Jersey cows in comparison to Holstein cows have been reported to have a lower risk and consequently the prevalence of mastitis (Iqbal & Siddique, 1999; Kelm *et al*., 2001; Youngerman *et al*., 2004; Biffa *et al*., 2005; Berry *et al*., 2007; Jingar *et al*., 2014a,b). Jingar *et al*. (2014b) reported the incidence of mastitis to increase significantly with increase in parity number in cattle species, but not in buffalo.

Dissimilarity in the character of the inflammatory response also influences the potency of the host to control intramammary invasions (Bannerman *et al*., 2008a). However, the immune response in the form of production of cytokines as a result of encountering mastitis pathogens was found to be similar (Bannerman *et al*., 2008a,b). Genetic basis of resistance to infectious diseases in animals depends on additive genetic variation that exists among animals and gives a differential response to various infectious challenges (Yañez *et al*., 2014). Therefore, it is imperative to study the genes responsible for disease resistance to mastitis associated pathogen and other pathogens. The aim of this study was to identify, evaluate and compare early phase immune response associated genes in peripheral blood mononuclear cells (PBMCs) isolated from blood of crossbred cattle, Tharparkar cattle and Murrah buffalo in response to *E. coli*'s LPS stimulation. It was done through the evaluation of relative expression of the transcripts by real-time PCR in order to explore out any breed-wise and species-wise difference(s) in resistance pattern towards mastitis.

Material and methods

Selection of animals for blood collection

All animals used in the experimentation study belonged to the Cattle and Buffalo Farm, ICAR-IVRI (Izatnagar, Bareilly, UP, India), and the animal experimentation was performed in strict compliance with the rules and regulations set by the Institutional Animal Ethics Committee (IAEC). Apparently healthy lactating crossbred Vrindavani cattle (rotational cross having 50–75% exotic inheritance viz. Holstein-Friesian, Jersey and Brown Swiss; with the rest coming from the native indigenous component Hariana cattle breed), Tharparkar cattle and Murrah buffaloes were taken for the study. The animals selected were mainly in their 3rd or 4th parity with no clinical history of mastitis and screening of milk samples was performed for different mastitis specific tests.

Procedure for initial screening of samples

The milk samples of clinical mastitis free animals were screened for California mastitis test (CMT) score and milk somatic cell count (SCC) to rule out any presence of subclinical mastitis. Milk samples of 60 animals (45 Crossbreds cattle, 15 Tharparkar cattle and 15 Murrah buffaloes) were screened by CMT and SCC. Among these, six animals from each group (crossbred, Tharparkar and Murrah) were selected as experimental animals, whose SCC were less than 200,000 cells/mL of milk and whose milk samples showed a CMT score of either 0 or 1. Blood (4 mL) from the six selected

experimental animals of each group was collected for PBMCs isolation by density gradient centrifugation method (Histopaque-1.083 g/mL, Sigma, Poole, Dorset, UK).

PBMCs culture and stimulation with LPS

The recovery and viability of the isolated PBMCs were checked by Countess™ Automated Cell Counter (Invitrogen, USA). Viable blood mononuclear cells obtained from each animal were plated at a density of 1×10^6 cells/mL (3×10^6 cells/well) into two rows of polystyrene culture plate wells. One row of culture plate wells were stimulated with *E. coli* outer membrane antigen LPS (Sigma-Aldrich, Saint Louis, USA) at a dose rate of 1 µg/mL of culture media with stimulation time of 6 h and another row was kept un-stimulated as a control for this study. The culture plates were kept at 37ºC with 5% CO_2 levels. *In vitro* stimulation of bovine PBMCs was done with LPS of *E. coli* at a pre-determined standardized dose as described by Doherty *et al*. (2013). The time period of 6 h was considered optimum for our study because most of the inflammatory cytokines after reaching an expression peak at about 4 to 6 h were either found to have no change or showed a decrease in the level of cytokines after 12 or 24 h of challenge (Strandberg *et al*., 2005). The supernatants obtained after centrifugation and separation of PBMCs were pooled for each animal and stored at -80ºC to be used for further estimation of IFN-γ protein by ELISA. All the supernatants were used within 48 h for ELISA protocol.

Total RNA extraction and qRT-PCR analysis

The differential mRNA expression levels of IL-10, *IFN-γ, IL-1β, IL-12β*, *CCL-5* genes in stimulated and unstimulated PBMCs were analysed by real-time PCR using Fast SYBR® Green dye (Applied Biosystems, Warrington, UK). The real-time PCR system (Step one plus, Applied Biosystems, Warrington, UK) was used with the already published and self-designed gene-specific primers mentioned in Table 1. RNA isolation from stimulated and unstimulated samples of PBMCs was carried out using RNeasy Plus Mini Kit (Qiagen) and the manufacturer's instructions. The extracted RNA was quantitated in NanoDrop ND 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The purity of RNA was evaluated by measuring the standard absorbance ratio for RNA (A260/A280). Synthesis of first strand cDNA was done using High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems, USA) following the manufacturer's instructions. Final Concentration of cDNA was adjusted to the level of 5 ng/µL. The thermal cycling program of the qPCR included one cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. A dissociation step was also included to confirm the specificity of amplification. Six biological samples with three technical replicates of each biological sample were used in the present study. A negative template control was also included in the qRT-PCR reaction.

The primers for the selected genes were either chosen from the reference paper(s) or designed with the help of Integrated DNA technologies (IDT) software Primer-Quest Tool (Coralville, IA, USA). For designed sets of primers, reaction efficiencies were calculated using a two-fold serial dilution of pooled cDNA. A standard curve was constructed by plotting average cycle thresholds (C_Ts) against $\log_{10}$(cDNA concentration/100). The line obtained from the slope (m) was then utilized to calculate the efficiencies of primers using the formula $10^{(-1/m)-1}$. Primers with efficiencies between 0.8 and 1.1

Table 1. Sequences of oligonucleotide primers.

Gene name	Primer sequence (5'-3')	Amplicon size (bp)	Accession number	Reference
IL-10	F: CAAAGCCATGAGTGAGTTTGAC R: CTGGAGGTCTTCTTCCCTAGA	99	EU276074.1	This work
IFN-γ	F: CTCTGAGAAACTGGAGGACTTC R: GTGACAGGTCATTCATCACTTTG	110	EF675629.1	This work
IL-1β	F: AGTGCCTACGCACATGTCTTC R: TGCGTCACACAGAAACTCGTC	114	NM_174093.1	Griesbeck-Zilch *et al*., 2009
IL-12β	F: GAAAAGAAACTCTTCATGGACCAAA R: GGACGTTGGCATCCTTGTG	64	XM_010807562.2	Taraktsoglou *et al*., 2011
CCL-5	F: CATGGCAGCAGTTGTCTTTATCA R: CTCTCGCACCCACTTCTTCTCT	76	NM_175827.2	Taraktsoglou *et al.*, 2011
β-Actin	F: CATCGGCAATGAGCGGTTC R: ACAGCACCGTGTTGGCGTAG	146	BC142413.1	This work

were considered satisfactory and were included in the study (Table 1). β-Actin was used as a reference gene for normalization of expression data (Doherty *et al*., 2013). The production of the melt curve as a result of the dissociation was observed for the presence of a single peak that shall represent a single PCR product for each gene amplified.

Estimation of IFN-γ by ELISA

IFN-γ levels were estimated in the supernatants obtained after centrifugation of the stimulated and unstimulated PBMCs via ELISA technique. The supernatant from six samples of each group of animals was collected. The samples were pooled together in a set of two for carrying out the ELISA protocol. Each of the pooled samples was tested in duplicate and IFN-γ was detected by the standard protocol (Cusabio, Hubei Province, China). The dilutions to which the sera were tested were up to 1:50. Standard and sample (100 µL) were added per well in 96-well plates, which were then incubated for 2 h at 37°C. Biotin antibody (100 µL) was added to each well and again incubated for 1 h. After washing three times, 100 µL of horseradish peroxidase (HRP) avidin was added to each well and incubated for 1 h. Plates were washed again five times following the standard procedure and then 90 µL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added to each well and incubated for 30 min and then 50 µL of stop solution was added. The optical density of each well was then determined within 5 min, using a microplate reader set at 450 nm.

Statistical analysis

qRT-PCR data were converted to $\log_2$ fold changes relative to control unstimulated samples using the $2^{-\Delta\Delta CT}$ method. Comparative cycle threshold (C_T) method (Schmittgen & Livak, 2008) was used for analysis of the generated data. The non-parametric Mann-Whitney U Test was performed to determine significant differences between ΔC_{TS} of the analyzed groups. Statistical analysis of the obtained ELISA data was done by using Tukey's Honest Significant Difference test by using SAS 9.2 version (SAS Inst., Cary, NC, USA).

Results

Cytokine mRNA expression of PBMCs against LPS challenge

PBMCs isolated from blood of the three groups (crossbred, Tharparkar and Murrah buffaloes) were stimulated with pre-determined standardized LPS dose of *E. coli* as a TLR-4 agonist in order to monitor the differential mRNA expression of *CCL5, IL-1β, IL-12β, IFN-γ* and *IL-10* genes with respect to unstimulated PBMCs. qRT-PCR technique was employed to establish any species-wise and breed-wise differential mode and intensity of immune response against a similar dose of antigen from the similar pathogen. Comparative cytokine profile analysis of *in vitro* stimulated PBMCs of the three groups with same pre-standardized LPS dose were made. As shown in Fig. 1, the expression level of *CCL5* gene was significantly up-regulated in stimulated PBMCs of crossbred ($p<0.01$) and Tharparkar ($p<0.01$) and was unaffected in PBMCs of Murrah when compared to un-stimulated control. The expression level of *IL-1β* gene was significantly ($p<0.01$) up-regulated, down-regulated and unchanged in stimulated PBMCs as compared to unstimulated PBMCs of crossbred cattle, Murrah buffaloes and Tharparkar cattle, respectively as shown in Fig. 1.

IL-12β gene showed significant upsurge ($p<0.01$) in stimulated PBMCs of Tharparkar and crossbred cattle but showed a significant decline ($p<0.01$) in stimulated PBMCs of Murrah buffaloes with respect to unstimulated control PBMCs (Fig. 1). In the case of *IFN-γ*, significantly higher ($p<0.05$) expression, and

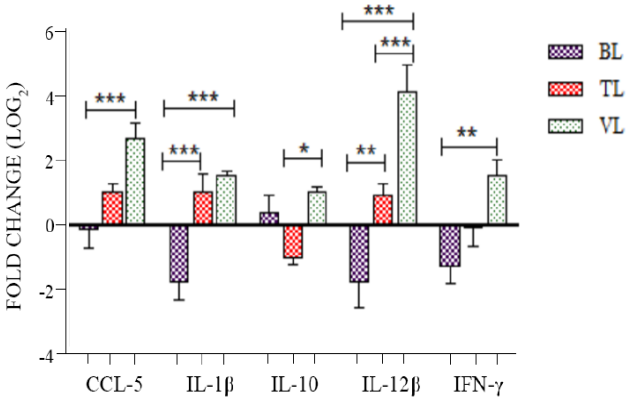

Figure 1. Relative fold change expression of genes in Murrah (BL), Tharparkar (TL), and crossbred (VL), respectively. One way ANOVA was performed to determine the significant diFferences between ΔC_{TS} of BL, TL, and VL for *CCL-5, IL-1β, IL-10, IL-12β* and *IFN-γ* gene respectively. *, **, *** indicate significance at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. The non-parametric Mann-Whitney U Test was performed to determine the significant differences between ΔC_{TS} of the challenged groups (Murrah, Tharparkar, and crossbred) with its own control. The figure depicts –0.11, 1.05 ($p<0.01$) and 2.72 ($p<0.01$) $\log_2$ fold difference of CCL5; –1.75 ($p<0.01$), 1.08 and 1.55 ($p<0.01$) $\log_2$ fold difference of IL-1β; 0.40, –1 ($p<0.01$) and 1.06 ($p<0.01$) $\log_2$ fold difference of IL-10; –1.76 ($p<0.01$), 0.96 ($p<0.01$) and 4.15 ($p<0.01$) $\log_2$ fold difference of IL-12β and –1.22 ($p<0.05$), –0.03 and 1.58 ($p<0.05$) $\log_2$ fold difference of IFN-γ mRNA expression respectively of the analysed group with its control.

significantly lower (p<0.05) expression and unchanged expression was respectively observed in stimulated PBMCs of crossbred cattle, Murrah buffaloes and Tharparkar cattle as shown in Fig. 1. The expression level of *IL-10* was significantly higher (p<0.01), lower (p<0.01) but unchanged in stimulated PBMCs of crossbred cattle, Tharparkar cattle and Murrah buffaloes, respectively (Fig. 1).

Comparative analysis of ΔC_{TS} of the *CCL5, IL-1β, IL-12β, IFN-γ* and *IL-10* between crossbred, Tharparkar and Murrah revealed differential regulation of gene expression. Significant differences for *CCL5, IL-1β, IL-12β* and *IFN-γ* and non-significant difference for the *IL-10* gene were observed between Murrah and crossbred cattle (Fig. 1). Tharparkar samples showed a significant difference for only *IL-12β* and *IL-10* genes when compared with crossbred ones (Fig. 1). On the other hand, the comparison between Murrah and Tharparkar showed significant differences for *IL-1β* and *IL-12β* genes only (Fig. 1).

IFN-γ detection in PBMCs culture supernatant – ELISA approach

Tukey's HSD test revealed no significant difference in the level of IFN-γ in stimulated (LPS) and unstimulated groups of Tharparkar and Murrah PBMCs supernatant but showed a significant difference (p<0.01) in the stimulated group when compared with the unstimulated group of crossbred cattle (Fig. 2).

Discussion

Mastitis resistant pattern of the host depends upon infectious dose, route of infection and virulence of pathogen strain, immune status of the host, mode and intensity of the immune response of host against encountering pathogen, etc. Identification of differentially expressed genes in response to a pathogen may be useful to identify important markers which in turn can serve as an important tool for selection of animals showing more tolerance towards this complex polygenic trait (Fonseca *et al*., 2009). For the first time, this study was undertaken for Tharparkar cattle and Murrah buffaloes in order to identify differentially expressed genes in response to *E. coli* infection.

E. coli-LPS, a major outer membrane antigen, binds with CD14/TLR4/MD2 receptor complex and induces cytokine release in various types of cells (Gerold *et al*., 2007; Lu *et al*., 2008). *IFN-γ*, a pro-inflammatory cytokine secreted by immune cells acts as an important link between innate and adaptive immunity and is very important against the intracellular pathogens (Bannerman, 2009). Its concentration is known to increase in sera during coliform mastitis (Hisaeda *et al*., 2001). Un-altered and significantly down-regulated *INF-γ* transcripts levels in stimulated PBMCs of Tharparkar cattle and Murrah buffalo respectively, whereas, significantly higher *INF-γ* transcript levels in stimulated PBMCs of crossbred cattle at a similar stimulant dose of *E. coli* LPS, suggest that immune system of each breed of cattle and also among different species combat encountering pathogens in a different manner. Results of ELISA test of *INF-γ* done on PBMCs supernatants of the cultures also gave indications in support of the real-time PCR results. *INF-γ* greatly enhances the ability of macrophages and neutrophils to phagocytose and eliminate invading pathogens (Ellis & Beaman, 2004). It also increases the activity of major histocompatibility complex (MHC) class-I molecule and finally promotes cell-mediated

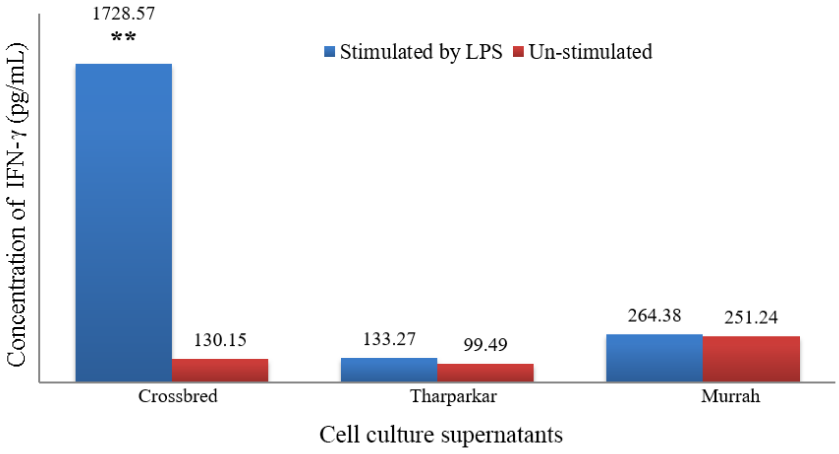

Figure 2. Average concentration of IFN-γ in stimulated (LPS) and unstimulated groups of crossbred, Tharparkar and Murrah cell culture supernatants. ** indicates significance at p<0.01.

immunity by helping cytotoxic T-cell recognition of antigens presented from foreign microbes (Schroder *et al*., 2004). Higher *IFN-γ* transcript levels were also detected in milk cells isolated from mammary glands of animals infected with *E. coli* (Lee *et al*., 2006).

On the other hand, mRNA expression of IL-12β displayed a significant increase in both crossbred and Tharparkar which was in concurrence with the result of Lee *et al*. (2006) but the *IL-12β* expression in Murrah PBMCs was significantly down-regulated. Expression in crossbred significantly varied with both Tharparkar and Murrah, and there was significant variation between the expression of *IL-12β* gene among Tharparkar and Murrah too. The results suggest that the immunocytes of all the three groups manifested a non-identical response when stimulated with the same dose of antigen from *E. coli*. *IL-12* produced by antigen-presenting cells such as macrophages after activation of PAMP and Damage Associated Molecular Pattern (DAMP) acts as a strict regulator of Th1 type of immune response and activates Natural Killer (NK) cells to secrete *IFN-γ*, which in turn further stimulates enhanced secretion of *IL-12* (Trinchieri, 2003; Vignali & Kuchroo, 2012; Pérez-Cordón *et al*., 2014).

IL-1β takes part in several activities such as cell proliferation, cell apoptosis, cell differentiation, leukocytes activation, systemic induction of fever and acute phase protein synthesis (Dinarello, 1996; Barksby *et al*., 2007). Up-regulated, unchanged and down-regulated expression levels of *IL-1β* gene in stimulated PBMCs as compared to unstimulated PBMCs of crossbred, Tharparkar and Murrah, respectively in response to a similar dose of *E. coli* may be reminiscent of differential inflammation regulatory pattern and activation of leucocytes in order to combat *E. coli* infection. Upsurge *IL-1β* level in response to LPS in crossbred concur the findings of Strandberg *et al*. (2005) who also observed increased levels of mRNA expression of *IL-1β* after 2-4 h of *in vitro* stimulation of bovine primary mammary epithelial cells by LPS.

CCL5 (Chemokine ligand 5), also known as RANTES, has a role in the inflammatory and immunoregulatory control and has been found to be important for chemotaxis and maintenance of inflammation inside bovine mammary gland (Ezzat Alnakip *et al*., 2014). *CCL5* acts as chemo-attractant for memory T-helper cells, blood monocytes and eosinophils (Song *et al*., 2015). Upsurge cytokine and chemokines expression level in response to LPS was also in accordance with the finding of Taraktsoglou *et al*. (2011). Challenge of bovine macrophages with LPS revealed a significant up-regulation of proinflammatory gene *CCL5* in PBMCs of crossbred and Tharparkar only. *CCL5* gene acts as an important chemokine during clinical mastitis cases and is involved in the recruitment of inflammatory cells and neutrophils to the site of inflammation (Taraktsoglou *et al*., 2011).

IL-10, a major anti-inflammatory cytokine, is produced in order to limit the excessive release of pro-inflammatory cytokines (*IL-1*, *IFN-γ*, *IL-12* and *TNF-α*), chemokines and eicosanoids to suppress Th-1 type response and enhance Th-2 type of response which in turn drive humoral immune response preferentially (Moore *et al*., 2001; Asadullah *et al*., 2003; Bannerman, 2009). Up-regulated, down-regulated and un-altered *IL-10* expression level in stimulated PBMCs of crossbred, Tharparkar and Murrah, respectively, in spite of similar stimulant dose of *E. coli*, may suggest species-wise and breed-wise different degree of the immune response against pathogen invasion. The result may be suggestive of the intensity of immune response against pathogen and the somewhat conclusive idea of mastitis resistant pattern which may vary according to breed and species. Animals with intramammary *E. coli* infection revealed an increased concentration of *IL-10* in milk (Bannerman *et al*., 2004).

Several studies suggest the role of a proper balance between different pro-inflammatory cytokines and anti-inflammatory cytokines in combating mastitis caused by the invasion of a variety of bacterial organisms (Stelwagen *et al*., 2009). *E. coli* infection leading to clinical mastitis causes high up-regulation of pro-inflammatory cytokines which may cause tissue damage or even death of the animals by inducing severe clinical mastitis and fever. A higher level of pro-inflammatory cytokines can be counterbalanced by an increase in the level of anti-inflammatory cytokine-like *IL-10*, so administration of such anti-inflammatory cytokines can be beneficial to counteract the effect of pro-inflammatory cytokines (Bannerman, 2009). It has been documented that cattle shows substantial genetic variation for disease resistance including mastitis (Morris, 2007; Sender *et al*., 2013; Litwińczuk *et al*., 2015). Breed-wise differences in mastitis occurrence have also been reported by Nóbrega & Langoni (2011). Crossbred stimulated PBMCs culture showed higher expression levels of *CCL5, IL-1β, IL-12β, IFN-γ* and *IL-10* with respect to unstimulated culture. Tharparkar stimulated PBMCs showed a significant upsurge in *CCL5* and *IL-12β* expression level only but lower expression level of these genes in comparison to crossbred, along with downturn of *IL-10* expression and non-significant alteration in *IL-1β* and *IFN-γ* expression level as compared with unstimulated PBMCs. The results give an indication that similar doses of the lipopolysaccharide were not able to induce a similar increase in response of pro-inflammatory cytokines in Tharparkar in comparison to crossbred and the

expression of anti-inflammatory cytokine thus showed a downturn in Tharparkar as it was not needed to control the over-expression of pro-inflammatory cytokines. The unchanged expression of *IL-1β* gene in Tharparkar PBMCs also gave a sign of positive immune response as the excessive release of *IL-1β* has the ability to induce vascular leakage, shock and multi-organ failure. This may also indicate a more balanced immune response of Tharparkar in comparison to crossbred. While Murrah stimulated PBMCs exhibited significant recession in mRNA level of *IL-1β, IL-12β* and *IFN-γ*, but non-significant alteration in *CCL5* and *IL-10* mRNA level as compared with unstimulated PBMCs. Our findings reaffirm the previous findings of Dewangan *et al*. (2015) and Panigrahi *et al*. (2016), which also suggested differential expression gene pattern in immune-related genes of PBMCs of crossbred, Tharparkar cattle and Murrah buffalo after stimulation with similar dose of *T. annulata* sporozoites, which may account for difference in disease resistance pattern to *T. annulata* infection among different species and breed. Bannerman *et al*. (2008a,b) working on a comparison of Holstein and Jersey innate immune response by intramammary infection of *E. coli* and *S. aureus*, were not able to find any differences between these two breeds and reported a highly conserved innate immune response of these breeds. However, Sulabh *et al*. (2018) reported the differential response of mRNA transcript of cytokines between stimulated PBMCs culture of crossbred, Tharparkar cattle and Murrah buffalo when they were stimulated with the same dose of peptidoglycan and lipoteichoic acid obtained from *S. aureus*. Differences in innate immune responses against various bacterial infections have also been reported by Fonseca *et al*. (2009) and Gibson *et al*. (2016).

Our results further showed the contrasting differences in the expression level of *IFN-γ, IL-1β* and *IL-12β* genes of Murrah in comparison to crossbred. This may suggest that PBMCs of Murrah may require a higher dose of antigen challenge. It also gave an indication that immunocytes of Murrah may be refractory to lower dose of antigen challenge and may require a higher number of *E. coli* bacteria to elicit an inflammatory response or to induce disease in these animals in comparison to crossbred; this may be one of the prime reasons for the lower incidence of mastitis in buffaloes. Yu *et al*. (2010) used various concentrations (1 ng/mL to 1 µg/mL for polymorphonuclear leucocytes for 6 h) in which the results highlighted an increased response of gene expression with increasing dose. However, for mRNA expression of *GCP-2* and *IL-8* they also used an additional dose rate of 10 µg/mL (for 24 h), which showed an increase in the expression of the concerned genes. However, proteomic studies, post-transcriptional modification, epigenetic regulation and inclusion of a large number of animals in the study are also required to confirm conclusive idea for genetic markers of mastitis resistant pattern of host apart from monitoring of relative immune-related gene expression level with stimulating LPS dose of *E. coli*.

The results of this study suggest that the immunocytes of the three groups manifested non-identical response when stimulated with the same dose of antigen from *E. coli*. It points towards the presence of differential immune response among different breeds/species, which have evolved as a result of evolutionary adaptation to a particular environment or specific man-made selection(s) that was responsible for the creation of different species/breeds of animals. This possibly indicates the role of the immune system in causing the differential outcome of a disease in response to the pathogen invasion.

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References

Asadullah K, Sterry W, Volk HD, 2003. Interleukin-10 therapy-Review of a new approach. Pharmacol Rev 55: 241-269. https://doi.org/10.1124/pr.55.2.4

Aslantaş O, Demir C, 2016. Investigation of the antibiotic resistance and biofilm-forming ability of Staphylococcus aureus from subclinical bovine mastitis cases. J Dairy Sci 99 (11): 8607-8613. https://doi.org/10.3168/jds.2016-11310

Bannerman DD, 2009. Pathogen-dependent induction of cytokines and other soluble inflammatory mediators during intramammary infection of dairy cows. J Anim Sci 87: 10-25. https://doi.org/10.2527/jas.2008-1187

Bannerman DD, Paape MJ, Lee JW, Zhao X, Hope JC, Rainard P, 2004. Escherichia coli and Staphylococcus aureus elicit differential innate immune responses following intramammary infection. Clin Diagn Lab Immunol 11: 463-472.

Bannerman DD, Kauf ACW, Paape MJ, Springer HR, Goff JP, 2008a. Comparison of Holstein and Jersey innate immune responses to Escherichia coli intramammary infection. J Anim Sci 91: 2225-2235.

Bannerman DD, Springer HR, Paape MJ, Kauf ACW, Goff JP, 2008b. Evaluation of breed-dependent differences in the innate immune responses of Holstein and Jersey

cows to Staphylococcus aureus intramammary infection. J Dairy Res 75: 291-301. https://doi.org/10.1017/S0022029908003427

Barksby HE, Lea SR, Preshaw PM, Taylor JJ, 2007. The expanding family of interleukin-1 cytokines and their role in destructive inflammatory disorders. Clin Exp Immunol 149: 217-225. https://doi.org/10.1111/j.1365-2249.2007.03441.x

Berry DP, Lee JM, Macdonald KA, Stafford K, Matthews L, Roche JR, 2007. Associations among body condition score, body weight, somatic cell count, and clinical mastitis in seasonally calving dairy cattle. J Dairy Sci 90: 637-648. https://doi.org/10.3168/jds.S0022-0302(07)71546-1

Biffa D, Debela E, Beyene F, 2005. Prevalence and risk factors of mastitis in lactating dairy cows in southern Ethiopia. Int J Appl Res Vet Med 3: 189-198.

Detilleux JC, Koehler KJ, Freeman AE, Kehrli ME, Kelley DH, 1994. Immunological parameters of periparturient Holstein cattle: Genetic variation. J Dairy Sci 77: 2640-2650. https://doi.org/10.3168/jds.S0022-0302(94)77205-2

Dewangan P, Panigrahi M, Kumar A, Saravanan BC, Ghosh S, Asaf VNM, Parida S, Gaur GK, Sharma D, Bhushan B, 2015. The mRNA expression of immune-related genes in crossbred and Tharparkar cattle in response to *in vitro* infection with Theileria annulata. Mol Biol Rep 42 (8): 1247-1255. https://doi.org/10.1007/s11033-015-3865-y

Dinarello CA, 1996. Biologic basis for interleukin-1 in disease. Blood 87: 2095-2147.

Doherty R, O'Farrelly C, Meade KG, 2013. Epigenetic regulation of the innate immune response to LPS in bovine peripheral blood mononuclear cells (PBMC). Vet Immunol Immunopathol 154: 102-110. https://doi.org/10.1016/j.vetimm.2013.05.004

Ellis TN, Beaman BL, 2004. Interferon-gamma activation of polymorphonuclear neutrophil function. Immunol 112: 2-12. https://doi.org/10.1111/j.1365-2567.2004.01849.x

Ezzat Alnakip ME, Quintela-Baluja M, Böhme K, Fernández-No I, Caamaño-Antelo S, Calo-Mata P, Barros-Velázquez J, 2014. The immunology of mammary gland of dairy ruminants between healthy and inflammatory conditions. J Vet Med 2014: 659801.

Falconer DS, Mackay TFC, 1996. Threshold characters. Introduction to quantitative genetics. Longman: Harlow, Essex, UK, pp: 299-311.

Fonseca I, Silva PV, Lange CC, Guimarães MF, Weller MM, Sousa KR, Lopes PS, Guimarães JD, Guimarães SE, 2009. Expression profile of genes associated with mastitis in dairy cattle. Genet Mol Biol 32 (4): 776-781. https://doi.org/10.1590/S1415-47572009005000074

Gerold G, Zychlinsky A, de Diego JL, 2007. What is the role of Toll-like receptors in bacterial infections? Semin Immunol 19: 41-47. https://doi.org/10.1016/j.smim.2006.12.003

Gibson AJ, Woodman S, Pennelegion C, Patterson R, Stuart E, Hosker N, Siviter P, Douglas C, Whitehouse J, Wilkinson W, Pegg SA, Villarreal-Ramos V, Werling D, 2016. Differential macrophage function in Brown Swiss and Holstein Friesian cattle. Vet Immunol Immunopathol 181: 15-23. https://doi.org/10.1016/j.vetimm.2016.02.018

Griesbeck-Zilch B, Osman M, Kühn C, Schwerin M, Bruckmaier RH, Pfaffl MW, Hammerle-Fickinger A, Meyer HH, Wellnitz O, 2009. Analysis of key molecules of the innate immune system in mammary epithelial cells isolated from marker-assisted and conventionally selected cattle. J Dairy Sci 92 (9): 4621-4633. https://doi.org/10.3168/jds.2008-1954

Heringstad B, Klemetsdal G, Steine T, 2007. Selection responses for disease resistance in two selection experiments with Norwegian red cows. J Dairy Sci 90: 2419-2426. https://doi.org/10.3168/jds.2006-805

Hisaeda K, Hagiwara K, Eguchi J, Yamanaka H, Kirisawa R, Iwai H, 2001. Interferon-gamma and tumor necrosis factor alpha levels in sera and whey of cattle with naturally occurring coliform mastitis. J Vet Med Sci 63: 1009-1011. https://doi.org/10.1292/jvms.63.1009

Iqbal J, Siddique M, 1999. Some epidemiological aspects of mastitis in cows and biocharecterization of isolated *Staphylococci*. Pak Vet J 19 (3): 149-154.

Jingar SC, Mehla RK, Singh M, 2014a. Climatic effects on occurrence of clinical mastitis in different breeds of cows and buffaloes. Arch Zootec 63 (243): 473-482. https://doi.org/10.4321/S0004-05922014000300008

Jingar SC, Mehla RK, Singh M, Kumar A, Kantwa SC, Singh N, 2014b. Comparative study on the incidence of mastitis during different parities in cows and buffaloes. Ind J Anim Res 48 (2): 194-197. https://doi.org/10.5958/j.0976-0555.48.2.040

Kelm SC, Freeman AE, Kehrli Jr, ME, 2001. Genetic control of disease resistance and immune responsiveness. Vet Clin North Am Food Anim Pract 17 (3): 477-493. https://doi.org/10.1016/S0749-0720(15)30002-5

Lee JW, Bannerman DD, Paape MJ, Huang MK, Zhao X, 2006. Characterization of cytokine expression in milk somatic cells during intramammary infections with Escherichia coli or Staphylococcus aureus by real-time PCR. Vet Res 37: 219-229. https://doi.org/10.1051/vetres:2005051

Litwińczuk Z, Król J, Brodziak A, 2015. Factors determining the susceptibility of cows to mastitis and losses incurred by producers due to the disease - A review. Ann Anim Sci 15 (4): 819-831. https://doi.org/10.1515/aoas-2015-0035

Lu YC, Yeh WC, Ohashi PS, 2008. LPS/TLR4 signal transduction pathway. Cytokine 42: 145-151. https://doi.org/10.1016/j.cyto.2008.01.006

Mogensen TH, 2009. Pathogen recognition and inflammatory signalling in innate immune defenses. Clin Microbiol Rev 22 (2): 240-273. https://doi.org/10.1128/CMR.00046-08

Moore KW, Malefyt RW, Coffman RL, O'Garra A, 2001. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 19: 683-765. https://doi.org/10.1146/annurev.immunol.19.1.683

Morris CA, 2007. A review of genetic resistance to disease in Bos taurus cattle. Vet J 174 (3): 481-91. https://doi.org/10.1016/j.tvjl.2006.09.006

Nóbrega DB, Langoni H, 2011. Breed and season influence on milk quality parameters and in mastitis occurrence. Pesq Vet Bras 31 (12): 1045-1052. https://doi.org/10.1590/S0100-736X2011001200002

Panigrahi M, Kumar A, Bhushan B, Ghosh S, Saravanan BC, Sulabh S, Parida S, Gaur GK, 2016. No change in mRNA expression of immune-related genes in peripheral blood mononuclear cells challenged with Theileria annulata in Murrah buffalo (Bubalus bubalis). Ticks Tick Borne Dis 7 (5): 754-758. https://doi.org/10.1016/j.ttbdis.2016.03.006

Park YH, Joo YS, Park JY, San Moon J, Kim SH, Kwon NH, Ahn JS, Davis WC, Davies CJ, 2004. Characterization of lymphocyte subpopulations and major histocompatibility complex haplotypes of mastitis resistant and susceptible cows. J Vet Sci 5 (1): 29-39.

Pérez-Cordón G, Estensoro I, Benedito-Palos L, Calduch-Giner JA, Sitjà-Bobadilla A, Pérez-Sánchez J, 2014. Interleukin gene expression is strongly modulated at the local level in a fish-parasite model. Fish Shellfish Immunol 37: 201-208. https://doi.org/10.1016/j.fsi.2014.01.022

Rupp R, Boichard D, 2003. Genetics of resistance to mastitis in dairy cattle. Vet Res 34 (5): 671-688. https://doi.org/10.1051/vetres:2003020

Schmittgen TD, Livak KJ, 2008. Analyzing real-time PCR data by the comparative Ct method. Nat Protoc 3: 1101-1108. https://doi.org/10.1038/nprot.2008.73

Schroder K, Hertzog PJ, Ravasi T, Hume DA, 2004. Interferon-gamma: An overview of signals, mechanisms and functions. J Leukoc Biol 75: 163-189. https://doi.org/10.1189/jlb.0603252

Sender G, Korwin-Kossakowska A, Pawlik A, Hameed KG, Oprządek J, 2013. Genetic basis of mastitis resistance in dairy cattle-a review. Ann Anim Sci 13 (4): 663-673. https://doi.org/10.2478/aoas-2013-0043

Song G, Tarrant TK, White TF, Barrow DA, Santos CM, Timoshchenko RG, Hanna SK, Ramanathan RK, Lee CR, Bae-Jump VL, Gehrig PA, 2015. Roles of chemokines CCL2 and CCL5 in the pharmacokinetics of PEGylated liposomal doxorubicin *in vivo* and in patients with recurrent epithelial ovarian cancer. Nanomed-Nanotechnol 11 (7): 1797-807. https://doi.org/10.1016/j.nano.2015.05.007

Stelwagen K, Carpenter E, Haigh B, Hodgkinson A, Wheeler TT, 2009. Immune components of bovine colostrum and milk. J Anim Sci 87 (13): 3-9. https://doi.org/10.2527/jas.2008-1377

Strandberg Y, Gray C, Vuocolo T, Donaldson L, Broadway M, Tellam R, 2005. Lipopolysaccharide and lipoteichoic acid induce different innate immune responses in bovine mammary epithelial cells. Cytokine 31: 72-86. https://doi.org/10.1016/j.cyto.2005.02.010

Sulabh S, Panigrahi M, Ahmad SF, Varshney R, Verma A, Baba NA, Kumar S, Kumari S, Chauhan A, Kumar P Bhushan B, 2018. Peptidoglycan and lipoteichoic acid induces differential mRNA response of immune-related genes in PBMC of crossbred, Tharparkar cattle and Murrah buffalo. Anim Biotechnol. https://doi.org/10.1080/10495398.2018.1461633

Taraktsoglou M, Szalabska U, Magee DA, Browne JA, Sweeney T, Gormley E, MacHugh DE, 2011. Transcriptional profiling of immune genes in bovine monocyte-derived macrophages exposed to bacterial antigens. Vet Immunol Immunopathol 140: 130-139. https://doi.org/10.1016/j.vetimm.2010.12.002

Trinchieri G, 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity Nat Rev Immunol 3: 133-146. https://doi.org/10.1038/nri1001

Vignali DA, Kuchroo VK, 2012. IL-12 family cytokines: immunological playmakers. Nat Immunol 13: 722-728. https://doi.org/10.1038/ni.2366

Xiao HB, Wang CR, Liu ZK, Wang JY, 2015. LPS induces pro-inflammatory response in mastitis mice and mammary epithelial cells: Possible involvement of NF-κB signaling and OPN. Pathol Biol (Paris) 63 (1): 11-16. https://doi.org/10.1016/j.patbio.2014.10.005

Yáñez JM, Houston RD, Newman S, 2014. Genetics and genomics of disease resistance in salmonid species. Front Genet 2014 (5): 415.

Youngerman SM, Saxton AM, Oliver SP, Pighetti GM, 2004. Association of CXCR2 polymorphisms with subclinical and clinical mastitis in dairy cattle. J Dairy Sci 8 (7): 2442-2448. https://doi.org/10.3168/jds.S0022-0302(04)73367-6

Yu C, Shi ZR, Chu CY, Lee KH, Zhao X, Lee JW, 2010. Expression of bovine granulocyte chemotactic protein-2 (GCP-2) in neutrophils and a mammary epithelial cell line (MAC-T) in response to various bacterial cell wall components. Vet J 186 (1): 89-95. https://doi.org/10.1016/j.tvjl.2009.07.012

Zanella R, 2016. Genomic tools and animal health. Vet Sci 3 (3): 21. https://doi.org/10.3390/vetsci3030021