JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2015. Vol. **9**(2), p. 1015-1023

Differential Toll-Like Receptor and Cytokine Gene Expression Profiles in Natural *Caprine Brucellosis*

Vikas K. Singh[1]*, Vivek K.Gupta[2], N. Shivasharanappa[2], Ajay Singh[2], Sushant Bhat[1], Rakesh Kumar[3] and K. Gururaj[2]

[1]Immunology Section, Indian Veterinary Research Institute, Izatnagar -243 122, India.
[2]Animal Health Division, Central Institute for Research on Goats, Makhdoom, Mathura 281122, India.
[3]Dairy Cattle Breeding Division, National Dairy Research Institute, Karnal - 132001, India.

(Received: 19 March 2015; accepted: 03 May 2015)

Initial host defense to bacterial infection is executed by innate immunity, and therefore the main aim of this study was to investigate the role of Toll-like receptors (TLR2, TLR4 & TLR9) along with IL12 and IFNγ during natural *Brucella melitenis* infection in goats. Goats which were recently aborted divided in two groups as normal and infected for *B. melitensis* based on serology, culture and PCR. The tissue samples were collected from mammary gland (MG), supra mammary lymph nodes (SMLN) and uterus from both groups after euthenization. Total RNA was extracted from collected tissues and cDNA was synthesized and amplified by Real time PCR. The results showed higher mRNA expression of TLR4, TLR9, IFN-γ and IL12 in SMLN and MG as compared to uterus of infected goats. Although, TLR2 expression was found lower in all the three tissues. This indicates that TLR4, TLR9, IFN-γ and IL12 play important role in disease resistance and its agonist can be used along with vaccine to enhance the immune response. Moreover, elevated level of IFN-γ and IL12 may be helpful in early diagnosis of brucellosis in goats.

Key words: Goat, Brucellosis, Toll like receptors, Cytokines.

Brucellosis is one of the common diseases of goats mainly caused by *Brucella melitensis*, although this pathogen may also infect cattle and other ruminants. The *B. melitensis* causes abortion and infertility in goats and also various chronic zoonotic infections in humans (Corbel, 1997; Smith and Ficht, 1990). The virulence of *Brucella* spp. is mostly dependent on its ability to enter and intracellular survival or multiplication in host cells (Boschiroli *et al.*, 2002). The localization within cell implies that the immunity against *Brucella* requires a cell-mediated immune response, which makes the Th1 arm of the response very crucial for controlling the infection (Splitter *et al*., 1996). The protection against this infection requires a long-lived cellular immune response, depending on the processing of the bacteria by macrophages (Araya *et al*., 1989; Baldwin and Winter, 1994). In addition, initial host defense against bacterial infection is executed by innate immunity stimulated by pathogen- associated molecular patterns (PAMP), conserved molecular structures common to different groups of pathogens that are recognized by host receptors known as pattern recognition receptors (PRRs) (Medzhitov and Janeway, 1997).

Toll like receptors (TLRs) are one of the important PRRs that play a key role in innate immunity. TLRs recognize microbial markers namely protein, carbohydrate, lipid, nucleic acids and/or their combinations in an efficient, non-self-reactive means to initiate a complex signaling cascade to

* To whom all correspondence should be addressed.
Tel: +91-8871688695;
E-mail: vikas4vet@gmail.com

activate a wide variety of transcription factors and inflammatory cytokines (Takeda and Akira, 2003). These cell surface molecules also activate complement, phagocytosis, inflammation and apoptosis in response to pathogen detection (West *et al*., 2006) which finally culminate in the initiation of adaptive immunity through the induction of pro-inflammatory mediators (Medzhitov and Janeway, 1997). Phylogeny analysis of the TLRs of goats with those of other species revealed a typical pattern wherein the TLRs 1, 6 and 10 and TLRs 7, 8 and 9 were clustered as single families, respectively (Raja *et al*., 2011). It has been proven that TLRs 7, 8 and 9 are endosomal while TLRs 1, 6 and 10 function by forming heterodimers for TLR ligand binding. The phylogenetic analysis based on only the LRR also revealed similar patterns although this was more likely to predict the grouping based on ligand binding properties (Werling *et al*., 2009). Oliveira *et al*. (2010) reported a role for TLR2 and TLR4 on *Brucella abortus* signaling but only observed the involvement of TLR4 in resistance (Campos *et al*., 2004). Barquero-Calvo *et al*. (2007) observed that TNF-α secretion in macrophages culture infected with *B. abortus* seems to depend somewhat on TLR2 and TLR4, but the signaling by these receptors does not affect the intracellular replication of this pathogen. However, TLR induced cytokines play crucial roles in enhancement of immune responses such as by linking innate and adaptive immunity and to potentiate the immune response among immune-associated cells. Cytokines, regarded as key players in brucellosis, are IL-12, IFN-γ, and TNF-α. IL-12 is a key cytokineproduced by B cells and macrophages and leads Th1 immune responses in the host that will ultimately induce the secretion of IFN-γ from T cells (Ko*et al*., 2002; Zhan and Cheers, 1995). Recently, all the ten TLR genes (TLR 1-10) of goat have been characterized (Raja *et al*., 2011); however, the information on differential expression of TLRs in caprine brucellosis is not available. Role of TLR and TLR- mediated cytokines in brucellosis in cattle have been documented (Oliveira *et al*., 2010), but there is dearth of information on TLR and cytokine gene expression profiles and their contribution to innate immunity in brucellosis in goats. Hence the more knowledge of mRNA expression of TLRs and their cytokines in caprine brucellosis is needed to understanding of innate immunity, development of effective vaccine and determination of cytokine markers for the early diagnosis of caprine brucellosis.

MATERIALS AND METHODS

Animals

Recently aborted goats (5) and normal goats (5) were procured from the Central Institute for Research on Goats, Makhdoom, India and screened for brucellosis by serology, culture and PCR.The aborted goats which were found *B. melitensis* positive by serology, culture and PCR taken as infective while normal goats found negative were taken as control.

Standard agglutination test (SAT)

The standard tube agglutination test was performed as per the protocol given by Wright and Smith (1987). Briefly, blood was collected from normal control goats and aborted goats immediately after abortion. Made two fold serial dilution of serum by adding 200 µl of serum in 800µl of PBS in first tube and transferred 500µl to 2nd and 3rd tube and in last 500 µl discarded. The dilution of serum was 1:10, 1:20 and 1:40 in 1st, 2nd and 3rd tubes respectively. Then 500 µl antigens (*B. melitensis* plane antigen) added in all tubes. All tubes were kept at 37°C for 24 hr. in incubator. The 'mantle' pattern of cell sediment was observed at or above 1:40 titer, it was considered as an indication of infection while a 'button' pattern was considered as negative.

Culture of *B. melitensis*

Vaginal swabs were collected from the aborted goats just after abortion with the help of clean and sterile swabs. For isolation of *B.melitensis*, vaginal swabs were inoculated by spreading on plates of sterile *Brucella* agar with hemin and vitamin k_1 media (Hi Media, India) and incubated at 37°C for 48 hr. The plates were observed at every 24 hr. for presence of the growth. After the growth, the colonies suspected for *Brucella* were picked up and transferred to another *Brucella* agar with hemin and vitamin k1 plates and incubated at 37°C for 2 days to obtain pure culture.

Polymerase chain reaction (PCR)

Extraction of DNA from suspected colonies was done by using MDI kit (Advanced

micro device Pvt. Ltd., India). The DNA isolated from colonies was used for polymerase chain reaction for the amplification of 16S rRNA&Omp 31 genes for the confirmatory identification of *B.melitensis*.The published primers were used to amplify 16S r RNAFP 5-AGAGTTTGATCCT GGCTCAG-3(Weisburg*et al.*,1991)and RP 5-ACGGCTACCTTGTTACGACTT-3 and Omp 31 FP 5- TGACAGACTTTTTCGCCGAA-3 & RP 5-TATGGATTGCAGCACCGC-3(Vizcaino *et al.*,1996).The reaction was carried out in final reaction volume of 25 µl in thermal cycler (Techne, TC 4000) with 2µl of template DNA along with 1µl of each type of primer (10pmol/µl) with 1µlTaq PCR master mix (2x) (Qiagen,USA). The cycling conditions were as follows – an initial denaturation of 94°C for 5min was given, followed by next 30 cycles at 94°C on 1 min (denaturation), annealing at 54°C for 1.5 min for 16S rRNA and 58°C for 1 min for Omp 31 and extension at 72°C for 1.5min (16S rRNA) & 1 min (Omp 31) and atlast final extension was given at 72°C for 10 min.The PCR products were run on 1.5% agarose gels containing 0.5µg of ethidium bromide per ml.

Collection of samples

Tissues samples namely mammary gland (MG), uterus and supra mammary lymph nodes (SMLN) were collected from infected and control goats immediately after humane sacrifice. The collected tissue sample first dipped into RNA - *later* (Sigma, USA) and stored in sterile tubes and transported to the laboratory for the extraction of total RNA.

RNA isolation, cDNA synthesis

Total RNA was extracted by using TRI reagent (Sigma, USA) following the manufacturer's protocol.The concentration and purity of the isolated RNA were determined by ND-1000 Spectrophotometer (Thermoscientific, USA).The purity of extracted RNA was ranging between 1.8 and 2.1 (A260/A280). A total quantity of 2 µg total RNA was treated with 2µl of DNase I (Fermentas Life Sciences, India) prior to reverse transcription to remove any DNA contamination from extracted RNA.The reverse transcription was performed using M-MuLVReverse Transcriptase (Thermo scientific, India). Briefly, 2µg of total RNA was mixed with 1µl of oligo $(dT)_{18}$ in a 0.2 ml PCR tube and incubated at 65°C for 5 min to remove the secondary structures within the RNA. Then the tubes were snap chilled on ice to prevent formation of secondary structures. This was followed by adding of 4 µl 5X RT-Buffer, 2 µl of dNTP mix (10 mM each), 1µl RiboLockRNase Inhibitor (20 µg/µl),1µl Revert Aid M-MuLV Reverse Transcriptase enzyme (200 µg/µl) and final volume was made to 20µl with nuclease free water. The contents were mixed properly and incubated at 42°C for 1 h in a thermo cycler (Techne, TC 4000) for reverse transcription to occur. The reaction was terminated by heating at 90°C for 2 min to inactivate M-MuLVRT enzyme. The cDNA prepared was stored at– 80°C for further use.

Real time quantitative RT-PCR

The differential expression of TLR genes such as TLR2, TLR4 and TLR9, cytokine genes (IFN-γ and IL-12) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as endogenous control in different tissues such as uterus, SMLN and MG were carried out by Quantitative SYBR Green Real Time PCR in CF96X Touch Real-Time PCR detection system (Biorad, USA) by using specific primers (Table 1). All the reactions were set in 20 µl of reaction volume in 8 strip tubes in triplicate. In short, 2µl of cDNA template (50ng) for each sample was added in tubes in triplicates along with no template controls (NTC) containing 10µl hot startVeriQuest™FastSYBR®GreenqPCR Master Mix with Fluorescein (2X)(USB,Affymetrix, USA),1 µl (10 pmol) of forward and reverse primer. The thermal cycle conditions were: Hold at 50°C for 2 min,initial activation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 3 s, annealing at temperature as shown in Table1 for 30 s, extension at 72°C for 30s and for melt curve: incrementof temperature at rate of 0.5 for 0.05s from 65.0°C to 95.0°C.

Real time qRT-PCR calculations

Keeping GAPDH as the endogenous control, ΔCt values were calculated by subtracting GAPDH Ct values from the Ct values of a specific target cytokine for each sample. The average ΔCt for samples collected from normal tissues was taken as the calibrator group (ΔCt calibrator group) for each sample. Then ΔΔCt values were calculated by subtracting the average ΔCt for normal follicular ($\Delta Ct_{calibrator\ group}$) from ΔCt of normalized target values ($\Delta Ct_{target\ group}$). The relative fold change ($2^{-\Delta\Delta^{Ct}}$) expressions of TLRs andcytokines genes were compared relative to normal in *Brucella*

affected tissues samples using Mann–Whitney U-test as implemented in version 16.0 of the SPSS software package. The level of significance was set at $P < 0.05$.

RESULTS

Diagnosis of Brucellosis in aborted goats.

Only 5 aborted goats were found positive for brucellosis by serum agglutination test (SAT) (titer>1:40) while the vaginal swabs of only 4- goats were shown characteristics of *Brucella* like growth on sterile *Brucella* agar with hemin and vitamin k_1 media. The confirmatory identification of *B. melitensis* was done by amplification of 16S rRNA and Omp 31 gene by PCR. The 16S rRNA gene was amplified by PCR and 1412 bp band was observed on 1% agarose gel electrophoresis, which was corresponding to the length of target gene (Fig.1). A 720 bp (Omp 31)was observed the on 1.2% agarose gel electrophoresis (Fig.2). Out of 5 - DNA samples, only in 3 samples 16 S rRNA and Omp 31 genes were amplified, which indicated the presence of *B. melitensis.*

Comparison of TLR expression in different tissues of Brucella infected goats using qRT-PCR.

The mRNA expression for the three TLRs such as TLR2, TLR4 and TLR9 were detected by qRT–PCR.Variation between the affected tissues and normal tissues is revealed by expressing the corrected values as fold differences (Table 2, Fig. 3). The relative levels of TLR mRNA differed across the tissues analyzed. The TLR4 was least expressed in the uterus and highes t in the SMLN, with 5.35-fold greater and significant ($P<0.05$) expression. The uterus expressed the lowest level of TLR2, whilst theSMLN expressed the greater abundance but the TLR2 expression was found non-significant ($P>0.05$) among the tissues analyzed. The mammary gland significantly ($P<0.05$) expressed highest levels of TLR-9 mRNA while uterus expressed minimum as compared to normal tissues.

Comparison of cytokine expression in different tissues of Brucella infected goats using qRT–PCR.

Highly specific primers capable of amplifying the each cytokines in qPCR were used as shown in Table 1. The Fig. 4 and Table 3 show the expression level of each cytokines determined by qPCR in MG, SMLN and Uterus. Transcriptional expression of IFNγ and IL12 were detected in different tissues. The mRNA expression of IFNγ was higher and highly significant ($P<0.01$) in the MG and SMLN, whereas IL12 expression was found significantly ($P<0.05$) high in these tissues. The uterus showed comparatively lower cytokines expression than the MG and SMLN in caprine brucellosis.

Table 1. Primer sequences used for real time RT- PCR

Gene	Base sequence5' <—Sequence—>3'	Product size(bp)	Annealing temp. (°C)	Melt peak (°C)	References
TLR2	F:ACGACGCCTTTGTGTCCTC R:CCGAAAGCACAAAGATTT	192	52	81.5	Menzies *et al*., 2006
TLR4	F:ACTGACGGGAAACCCTATCC R:CAGGTTGGGAAGGTCAGAAA	208	52	82.5	Menzies *et al*., 2006
TLR9	F:CTCGTATCCCTGTCGCTGAG R:CACCTCCGTGAGGTTGTTGT	210	57	85.5	Menzies *et al*., 2006
IFNã	F:TTCCGGTGGATGATCTGC R:GAGAACCATTACATTGATGCTC	148	59	81.5	Tourais-Esteves *et al*., 2008
IL12	F:AACCTGCAACTGAGACCACT R:ATCCTTGTGGCATGTGACTT	186	59	79.5	Tourais-Esteves *et al*., 2008
GAPDH	F: CCTGGAGAAACCTGCCAAGT R: GCCAAATTCATTGTCGTACCA	200	60	81.5	Menzies *et al*., 2006

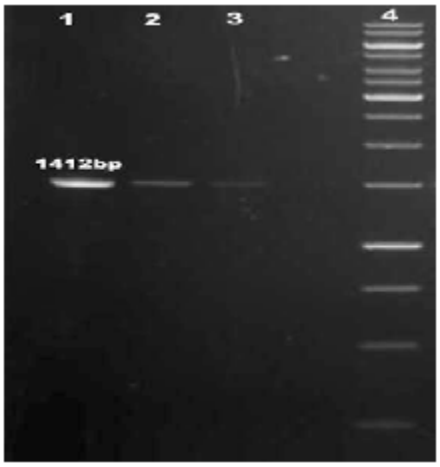
DISCUSSION

TLRs recognize pathogens and initiate development of an immune response by activation of different signaling pathways such as increase secretion of cytokine and chemokine and up-regulation of co-stimulatory molecules in APCs (Kawai and Akira, 2006). It is clear that activation of TLRs indirectly also stimulate the adaptive immune response (Iwasaki and Medzhitov, 2010). Moreover, T and B cell subsets have been reported to express various functional TLRs (Booth *et al*., 2011; Kulkarni *et al*., 2011; Wesch *et al*., 2011) that play significant role in controlling *Brucella* infection (Bertotto *et al*., 1993; Ottones *et al*., 2000). The importance of TLRs to limit the *Brucella* infection has been described in different species but not in goats. In current study, we found prominent contribution of TLR2, TLR4&TLR9 in controlling the infection in three tissues: MG, SMLN and Uterus of *Brucella* infected goats that form the interface between host and pathogen.

Table 2. Cytokines expression in different tissues of Brucellosis affected goats

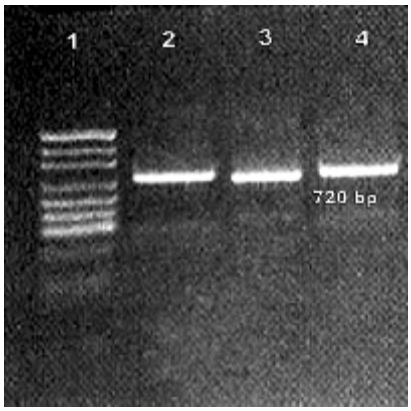
TLRs Genes	Tissues	Fold change ($2^{-\Delta\Delta Ct}$) Mean ± S.E.M	Change in expression
TLR2	Mammary glands	3.69±0.55[a]	Increase
	SMLN	4.83±0.40 [a]	Increase
	Uterus	2.91±0.53 [a]	Increase
TLR4	Mammary glands	4.55±0.56 [a]	Increase
	SMLN	5.35±0.88[b]	Increase
	Uterus	2.29±0.35 [a]	Increase
TLR9	Mammary glands	8.33±0.78 [b]	Increase
	SMLN	7.80±0.49 [a]	Increase
	Uterus	3.33±0.61 [a]	Increase

SEM: Standard Error of Mean
Means bearing at least one common superscript ([a, b]) do not differ significantly between tissues (P<0.05).



Lane 1, 2, 3: DNA from vaginal swabs of positive goats.
Lane 4: Standard DNA marker (100bp).

Fig. 1: PCR amplification of 16S rRNA gene for detection of *Brucella melitensis* from vaginal swabs of aborted goats.



Lane 1: Standard DNA Marker (100bp).
Lane 2, 3, 4:DNA from vaginal swabs of positive goats.

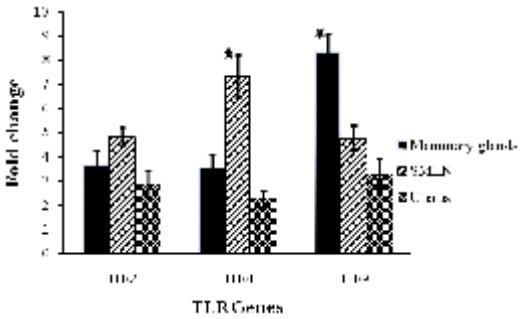
Fig.2: PCR amplification of Omp-31 gene for detection of *Brucella melitensis* from vaginal swabs of aborted goats.

Transcript expression of each of TLR2, TLR4 and TLR9 were confirmed in the MG, SMLN and Uterus of *Brucella* infected goats byqPCR. Oliveira *et al.*, 2012 reported the lipoprotein (Omp) especially L-Omp 19 lipoprotein of *B. abortus*is an important PAMPs candidate responsible for TLR 2 activation. The expression of TLR2 was found more in the SMLN, mammary gland than Uterusand it may be due to localization of *B. melitensis* in the SMLN and mammary gland.But overall expression of TLR2 was less as compared to the TLR4 and TLR9 expression in these tissues. This result suggests that the TLR2 doesn't play very important role in the host resistance to *B. melitensis* infection *in vivo*. Similar finding was reported by Campos *et al*., 2004; Weiss *et al*., 2005 regarding the TLR2 role in controlling *B. abortus* infection *in vivo* in mice. It is interesting that TLRs 4 and 9 are relatively more abundant in the mammary gland and SMLN than uterus suggesting the role of these TLRs in resistance to *B. melitensis*infection in goats. We found relatively lower TLR 4 expressions in these tissues as compared to the TLR9 expression indicating TLR 9 plays prominent role among TLRs in disease resistance. Similar result has been reported by the Copin *et al*., 2007; Oliveira *et al*., 2012 in *B. abortus* infections in mice. The TLR9 recognizes unmethylated CpG motifs of bacteria that are a potent stimulator of the host immune response (Hemmi *et al*. 2000). *Brucella melitensis* are the facultative intracellular microorganism and so that it'sunmethylated DNA

Table 3. Cytokines expression of different tissues of Brucellosis affected goats

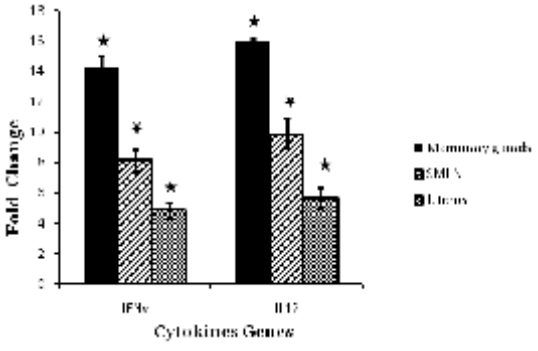
Cytokine Genes	Tissues	Fold change ($2^{-\Delta\Delta Ct}$) Mean ± S.E.M	Change in expression
IFN γ	Mammary gland	14.29 ± 0.76^{c}	Increase
	SMLN	8.15 ± 0.70^{b}	Increase
	Uterus	4.87 ± 0.53^{a}	
	Increase		
IL12	Mammary gland	16.04 ± 0.15^{c}	Increase
	SMLN	9.90 ± 0.99^{b}	Increase
	Uterus	5.66 ± 0.73^{a}	Increase

SEM: Standard Error of Mean
Means bearing at least one common superscript ($^{a, b, c}$) do not differ significantly between tissues (P<0.05).



★Significant (P<0.05)

Fig. 3. TLRs mRNA expression in Mammary Gland, Supramammary Lymph Node (SMLN) and uterus of *B. melitensis* infected goats. Normalized fold change expressions were assayed by real time PCR.The data are expressed as mean±SEM.



★ Significant (P<0.05)

Fig. 4. Cytokines mRNA expression in mammary gland, supramammary lymph node (SMLN) & uterus of *B. melitensis* infected goats. Normalized fold change expressions were assayed by real time PCR. The data are expressed as mean±SEM.

is easily recognized by theendosomal TLR9. The high expression of TLR 9 in mammary gland and SMLN could be dueto abundance of cells to express TLR9 including, monocytes, B cells and T cells along with high bacterial loadin these organs of infected goat. The relatively lower TLR4 expression observed in our study may be due to minor structural difference of lipid 'A'moiety of the LPS of *Brucella* elicits a reduced and delayed inflammatory response in the infected hosts compared with the endotoxins of other Gram-negative bacteria (Barquero-Calvo *et al*., 2007).

Cytokines play an important role in the clearance of *Brucella* infection especially Th1 cytokines like IFNγ, which activates the killing ability of macrophage. (Skendros & Boura, 2013). The cell mediated immunity in brucellosis consists of the interaction between T lymphocytes and macrophages.The macrophages express different types of TLRs, they recognize conserved microbial pattern and activate the secretion for various cytokines, which enhances expression of antigen-presentation, and co stimulatory molecules on APCs, and also help in the differentiation of Th cells into Th1 or Th2 subtypes (Skendros *et al*, 2011). The IL12 helps in the differentiation of Th cell into Th1 cells,resulting into more IFNγ production.

Interleukin 12(IL 12) and IFN γ are the most important cytokines which involve in the resistance against brucellosis (Ko*et al*., 2002). The interaction between these two cytokines determines the cell mediated immune response, which is essential for the removal of intracellular pathogens like *Brucella*. Interleukin 12 (IL-12) gene is a key cytokine produced by B cells and macrophages and leads Th1 immune responses in the host that will ultimately induce the secretion of IFN γ from Th1 cells. (Zhan and Cheers, 1998). In present study, we observed higher transcriptional expressionsof IL12 and IFN γ in MG and SMLN as compared to uterus in caprine brucellosis.It may be due to higher expression of TLR9, and more infiltration of leucocytes and macrophages leading to the production of IL-12 which drives the Th1 immune response (Macedo *et al.,* 2008).The uterus showed comparatively lower expression of IL12 and IFNγ might be due to more localization of *B. melitensis* in MG and SMLN as compared to uterus in caprine brucellosis. The similar higher IFNγ level were reported in murine spleenocytes (Ko *et al*., 2002 and Sathiyaseelan *et al.* 2006), bovine T cells (Weynants *et al*., 1998) and human PBMCs (Rafi ei *et al*., 2006 and Giambartolomei *et al*., 2002) after *in vitro* stimulation by *Brucella* antigens.So, from the present study it can be said that TLR4, TLR9, IL 12 and IFN γ play important role in the resistance against caprine brucellosis.

CONCLUSION

It is the first report of TLRs and cytokine genes expression quantified using real-time PCR in different tissues of natural brucellosis in caprine. Our findings suggest a significantly higher expression of IL12 and IFN γ mRNA in the MG, SMLNand uterus of caprine brucellosis, whereas significant up-regulation of TLR4 in SMLN and TLR9 inMG mRNA was observed. The transcriptional expression of TLR2 was found higher but non-significant in these tissues. Moreover the involvement of MG and SMLN was found to be greater than the role of uterus of caprine brucellosis. This indicates that these TLRs and cytokines gene may be used as adjuvant along with vaccine and also for accurate and early diagnosis of caprine brucellosis. However, further study is required to determine the threshold level of each TLRs and cytokine in biopsies of these tissues in large number of goats to establish such candidate gene(s) as marker of caprine brucellosis.

ACKNOWLEDGEMENTS

We thank Director, CIRG, Makhdoom and Director, IVRI, Izatnagar for providing necessary research facility.

REFERENCES

1. Araya, L. N., Elzer, P. H., Rowe, G. E., Enright, F.M., winter, A. J. Temporal development of protective cell-mediated and humoral immunity in BALB/c mice infected with *Brucella abortus*. *J. Immunol*., 1989; **143**: 3330–3337.
2. Baldwin, C. L., Winter, A. J. Macrophages and *Brucella*. *Immunol Ser*., 1994; **60**: 363–38.
3. Barquero-Calvo, E., Chaves-Olarte, E., Weiss,

D.S.*Brucellaabortus* uses a stealthy strategy to avoid activation of the innate immune system during the onset of infection. *PLoSOne.*, 2007; **2**:631.

4. Bertotto A., Gerli R., Spinozzi F., Muscat C., Scalise F., Castellucci G., Sposito M., Candio F., Vaccaro R. Lymphocytes bearing the gamma delta T cell receptor in acute *Brucella melitensis* infection. *Eur. J. Immunol.*, 1993; **23**:1177–1180.
5. Booth, J., Wilson, H., Jimbo, S., Mutwiri, G. Modulation of B cell responses by Toll-like receptors. *Cell Tissue Res*., 2011; **343**:131–140.
6. Boschiroli, M.L., Ouahrani-Bettache, S., Foulongne, V. The *Brucella suis*virB operonis induced intracellularly in macrophages. *ProcNatlAcad Sci.*, 2002; **99**: 1544-9.
7. Campos, M.A., Rosinha, G.M., Almeida, I.C. Role of Toll-like receptor 4 in induction of cell-mediated immunity and resistance to *Brucellaabortus* infection in mice. *Infect. Immun*., 2004; **72**:176-86.
8. Copin, R., De Baetselier, P.,Carlier, Y.MyD88-dependent activation of B220-CD11b+LY-6C+ dendritic cells during *Brucella melitensis* infection. *J. Immunol*., 2007; **178**:5182-91.
9. Corbel, M.J. Recent advances in brucellosis. *J. Med. Microbiol*., 1997; **46**:101-103.
10. Giambartolomei, G.H., Delpino, M.V., Cahanovich, M.E., Wallach, J.C., Baldi, P.C., Velikovsky,C.A.,Fossati, C.A., Diminished production of T helper 1 cytokines correlates with T cell unresponsiveness to *Brucella* cytoplasmic proteins in chronic human brucellosis. *J. infect. Dis.*, 2002; **186**: 252–259.
11. Hemmi,H.,Takeuchi,O.,Kawai,T.,Kaisho,T., Sato, S.,Sanjo, H.,Matsumoto, M.,Hoshino, K.,WagnerH., Takeda,K., Akira.S., A Toll-like receptor recognizes bacterial DNA. *Nature*, 2000; **408**:740–745.
12. Iwasaki, A., Medzhitov, R. Regulation of adaptive immunity by the innate immune system. *Science*., 2010; **327**: 291–295.
13. Kawai, T., Akira, S. TLR signaling. *Cell Death Differ.*, 2006; **13**:816–825.
14. Ko, J., Gendron-Fitzpatrick, A., Splitter, G.A. Susceptibility of interferon regulatory factor (IRF-1) and interferon consensus sequence binding protein (ICSBP) deficient mice to brucellosis. *J. Immunol*., 2002; **168**:2433–2440.
15. Kulkarni, R., Behboudi, S., Sharif, S.Insights into the role of Toll-like receptors in modulation of T cell responses. *Cell Tissue Res*., 2011; **343**:141–152.
16. Macedo, G.C., Magnani, D.M., Carvalho, N.B., Bruna-Romero, O., Gazzinelli, R.T.,Oliveira, S.C.Central role of MyD88-dependent dendritic cell maturation and proinflammatory cytokine production to control *Brucella abortus* infection.J. *Immunol*., 2008; **180**:1080–1087.
17. Medzhitov, R.,Janeway, C.A. Jr. Innate immunity: the virtues of a non-clonalsystem of recognition. *Cell*., 1997; **91**:295–298.
18. Menzies, M.,Ingham, A. Identification and expression of Toll-like receptors 1-10 in selected bovine and ovine tissues. *Vet. Immunol.Immunopathol*., 2006; **109**: 23–30.
19. Oliveira,S.C., Macedo,G.C.,deAlmeida,L.A., deOliveira,F.S.,Onate,A.,Giambartolomei,G.H., Cassataro,J. Recent Advances in Understanding Immunity against Brucellosis: Application for Vaccine Development. *The Open Veterinary Science Journal*., 2010; **4**:102-108.
20. Oliveira, S.C., deAlmeida, L.A., Carvalho, N.B., Oliveira, F.S., Lacerda, L.S. Update on the role of innate immune receptors during *Brucella abortus* infection. *Vet. Immunol. Immunopathol.*, 2012; **148**:129-135.
21. Ottones, F., Liautard, J., Gross, A., Rabenoelina, F., Liautard, J. P., Favero, J. Activation of human Vgamma Vdelta T cells by a *Brucellasuis* non-peptidic fraction impairs bacterial intracellular multiplication in monocytic infected cells. Immunology, 2000; **100**: 252–258.
22. Rafi ei, A., Ardestani, S.K., Kariminia, A., Keyhani, A., Mohraz, M., Amirkhani, A.Dominant Th1 cytokine production in early onset of human brucellosis followed by switching towards Th2 along prolongation of disease.*J. Infect.*, 2006; **53**:315–324.
23. Raja,A.,Vignesha,A.R., AnnMarya, B.,Tirumurugaana, K.G.,Dhinakar Raj, G.,Katariab,R., Mishra,B.P., Kumanan.K. Sequence analysisof Toll-likereceptorgenes1–10 of goat (*Capra hircus*). *Vet. Immunol. Immuno pathol*., 2011; **140**: 252–258.
24. Sathiyaseelan, J., Goenka, R., Parent, M. Treatment of *Brucella*susceptiblemice with IL-12 increases primary and secondary immunity. *Cell Immunol*., 2006; **243**:1-9.
25. Skendros, P., Pappas, G., Boura, P.Cell-mediated immunity in human brucellosis. *Microbes Infect*., 2011;**13**:134–142.
26. Skendros, P.,Boura, P. Immunity to brucellosis.*Rev. sci. tech. Off. int. Epiz.*, 2013; **32**:137-147.
27. Smith, L.D., Ficht, T.A. Pathogenesis of Brucella.*Crit. Rev. Microbial*., 1990; **7**: 209- 230.
28. Splitter, G., Oliveira, S., Carey, M., Miller, C., Ko, J., Covert, J. T lymphocyte mediated protection against facultative intracellular bacteria. *Vet Immunol Immunopathol*., 1996; **54**:

309–319.
29. Takeda, K., Kaisho, T., Akira, S. Toll-like receptors.*Annu. Rev. Immunol*., 2003; **21**:335.
30. Tourais-Esteves, I., Bernardet, N., Lacroix-Lamande, S., Ferret-Bernard, S., Laurent, F. Neonatal goats display a stronger TH1-type cytokine response to TLR ligands than adults. *Dev. Compa. Immunol*., 2008; **32**:1231–1241.
31. Vizcaino, N., Cloeckaert, A., Zygmunt, M.S.,Dubray, G. Cloning, nucleotide sequence, and expression of the *Brucellamelitensis*omp31 gene coding for an immunogenic major outer membrane protein. *Infect.immun.*, 1996; **64**: 3744-3751.
32. Weisburg, W.G, Barns, S.M, Pelletier, D.A, Lane, D.J. 16S ribosomal DNA for phylogenetic study. *J. Bacteriol*., 1991; **173**: 697–703.
33. Weiss, D.S., Takeda, K., Akira, S. MyD88, but not toll-like receptors 4 and 2, is required for efficient clearance of *Brucella abortus*. *Infect Immun*., 2005; **73**: 5137-43.
34. Werling, D., Jann, O.C., Offord, V., Glass, E.J., Coffey, T.J. Variation matters: TLR structure and species-specific pathogen recognition. *Trends Immunol*., 2009; **30**: 124–130.
35. Wesch, D., Peters, C., Oberg, H.H., Pietschmann, K., Kabelitz, D. Modulation of gamma-Delta T cell responses by TLR ligands. *Cell.Mol. Life Sci*., 2011; **68**: 2357–2370.
36. West, A.P., Koblansky, A.A.,Ghosh, S.Recognition and signaling by toll-like receptors.*Annu. Rev. Cell. Dev. Biol*., 2006; **22**: 409–437.
37. Weynants, V., Walravens, K., Didembourg, C., Flanagan, P., Godfroid, J.,Letesson, J.J. Quantitative assessment by flow cytometry of T-lymphocytes producing antigenspecific gamma-interferon in *Brucella* immune cattle. *Vet. Immunol.Immunopathol*., 1998; **66**: 309–320.
38. Wright,A.E., Smith, F. On the application of the serum test to the differential diagnosis of typhoid and Malta fever. *Lancet*., 1897; **1**: 656–9.
39. Zhan, Y., Cheers, C. Endogenous interleukin-12 is involved in resistance to *Brucellaabortus* infection. *Infect. Immun.*, 1995; **63**: 1387–1390.
40. Zhan, Y., Cheers, C. Control of IL-12 and IFN-ã production in response to live or dead bacteria by TNF and other factors. *J. Immunol*., 1998; **161**: 1447–1453.