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ASSOCIATION OF TOLL-LIKE RECEPTOR 4 GENE POLYMORPHISM WITH THE SEVERITY OF MASTITIS IN CROSSBRED DAIRY CATTLE

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ABSTRACT

Mastitis is a production disease with severe economic impact on the dairy industry worldwide. In the present study, we analyzed TLR4 gene in Jersey and Holstein Frisian (HF) crossbred cattle (n = 30 each) for genetic polymorphism and its association with the severity of mastitis. Animals were selected on history of the mastitis and screening by California mastitis test (CMT) and electrical conductivity (EC). The genetic polymorphism in Toll-like receptor (TLR4) gene was analyzed by PCR-RFLP in exon1 and exon2 using *Hae*III/*Dpn*I and *Hinf*I/*Tru*I enzymes, respectively. The screening of milk revealed that the normal animals with CMT negative or trace have EC value in the range of 3.7-4.9 mS cm^{-1}, subclinical mastitis with CMT 1 or 2 in the range of 5.2-5.7 $mScm^{-1}$ and clinical mastitis with CMT 3 or above in the range of 6.8-7.3 mS cm^{-1}. The exon1 exhibits polymorphism at *Hae*III site, but not at *Dpn*I site, with three prevalent genotypes in both the breeds. The exon2 revealed polymorphism at *Hinf*I site with three genotypes and at *Tru*I site having two genotypes in both the breeds. A significant association was observed between mastitis and polymorphism at *Hae*III site in exon1 and *Hinf*I site in exon2 of TLR4 gene in HF cross bred cattle only. The results demonstrated that TLR4 gene may be the candidate genetic biomarker for marker assisted selection (MAS) against mastitis in crossbred dairy cattle in Kashmir.

Key words: Bovine; Kashmir, mastitis; polymorphism; RFLP analysis, TLR4 gene

INTRODUCTION

Bovine mastitis, an inflammatory disease of the mammary gland, is characterized by physical, chemical, bacteriological changes in milk and pathological changes in glandular tissues of the udder (Sharma *et al.,* 2012). Mastitis can have infectious or non-infectious etiology that usually involves mammary parenchyma and affects the quality and quantity of milk (Bradley, 2002). Infectious agents are usually bacteria (*Staphylococcus* and *Streptococcus* species and many others) cause inflammatory reaction in the mammary gland (Sharma *et al.,* 2006) triggered by external (Pathogen Associated Molecular Patterns - PAMP) or internal (Damaged Associated Molecular Pattern - DAMP) stimuli. PAMPs include exo- and endo-toxins, polysaccharides, flagellin, nucleic acids and mannans of Gram +ve and Gram -ve bacteria as well as of some fungi and viruses (Brodsky *et al.,*

2009). Whereas, DAMPs are the molecules derived from mitochondria, nucleus, endoplasmic reticulum and other intracellular compartments released after cell death or injury (Krysko *et al.*, 2011). These molecular patterns are recognized by a class of membrane bound or cytosolic pattern recognition receptors (PRR) including Toll-like receptors (TLRs) and Nod-like receptors (NLRs).

TLRs are membrane-bound receptors that play central role in distinguishing different types of pathogens and drives the development of an appropriate local or systemic inflammatory response (Medzhitov *et al.*, 2009). In recent times, many studies have associated the susceptibility or resistance to mastitis in dairy animals to single nucleotide polymorphisms (SNP) within TLR genes (Werling and Jungi, 2003; Goldammer *et al*., 2004). Bovine TLR4 gene has been confirmed as a candidate gene for resistance against mastitis (Sharma *et al*., 2006; Wang *et al.*, 2007). TLR4 gene has been mapped to chromosome 8 and the whole gene is estimated to be approximately 11 kb consisting of three exons and two introns (McGuire *et al.*, 2005). The exon1 includes 1-95coding bases, exon2 consists of 96-260bases and exon3 has261-2526bases in coding region. On bacterial invasion a serum protein called lipopolysaccharide binding protein lipopolysaccharide (LBP) binds to LPS and delivers it to membrane-bound receptor CD14, expressed on the surface of macrophages and other inflammatory cells. After the activation of CD14, the complex CD14-LPS interacts with TLR4, which then translates the inflammatory signal from outside the cell to inside (Zheng *et al.*, 2006). TLR4 uses either Mal/TIRAP or MyD88 or TRAM and TRIF to signal to NF-κB or antiviral response mediated through interferon response factor 3 (IRF3), respectively (Fitzgerald *et al*., 2003). The transcription factor NF-κB or IRF3 stimulates transcription of pro-inflammatory cytokines and chemokines that finely regulate the systemic inflammatory events.

The SNPs in the coding region of TLR4 gene have been found associated with the susceptibility to inflammation, infection, neoplasms and trauma in human medicine; however their application in veterinary medicine is lacking. In the present study, we have investigated the polymorphism in TLR4 gene exon1 and exon2 by PCR-RFLP analysis and associated the polymorphism with the severity of mastitis in Jersey and HF crossbred cattle in Kashmir region.

MATERIALS AND METHODS

The present investigation was conducted on clinical cases attended at Clinical Complex and Division of Veterinary Biochemistry, FVSc & AH, Shuhama, Kashmir between June 2013 and April 2014. Cross bred animals of either Jersey or Holstein Frisian (HF) breed were divided into three groups (n = 10 each) *viz.*, normal, subclinical mastitis and clinical mastitis based on physical examination of animal and screening tests of milk *viz.*, California mastitis test (CMT) and electrical conductivity (EC). CMT was carried on milk samples as per the method described by Schalm *et al.*, (1971) and the reaction was scored as “zero”, “trace”, “l”, “2” and “3”.EC of milk samples was measured by digital electric conductivity meter (Eutech, Singapore) at 25°C immediately after the collection of milk as per the manufacturer`s instructions. The animal with EC of milk in the range of 4-5 mS cm^{-1} was assigned normal, 5-6 mS cm^{-1} subclinical mastitis and above 6mS cm^{-1} considered as clinical mastitis (Norberg *et al*., 2004).

Blood samples (5 mL) were drawn from jugular vein aseptically into EDTA-Vacutainer tubes (Biogene, USA) and stored at –20^{o}C until used. DNA was extracted from the blood samples using phenol-chloroform method as described by Sambrook *et al.*, (1989) and analyzed for quality and quantity by agarose gel electrophoresis and UV-spectrophotometry. The DNA samples with intact band on the gel without shearing and showing A_{260}nm/A_{280}nm ratio in the range of 1.7-1.9 were used for PCR-RFLP analysis.

Two region of TLR4 gene including exon1 (spanning 1-477bp) and exon2 (spanning 4987-5302bp) were amplified using specific-primers by polymerase chain reaction (PCR). The PCR was

carried out in 25 µL reaction volume in a thermo cycler (Eppendorf, Germany) using readymade master mix (Genei, Bangalore). The reaction consisted of initial denaturation of 4 min at 95°C followed by 35 cycles, each having denaturation at 94°C for 30 sec, annealing at 51.8°C (exon1) or 54.5°C (exon2) for 45 sec, extension at 72°C for 45 sec and the final extension at 72°C for 10 min. The PCR products were analyzed on 1.5% agarose gel electrophoresis along with DNA ladder (50 or 100bp). The sizes of amplicons were judged by comparing with the molecular size marker.

The sequence of forward and reverse primers for exon1 were 5' GGGTATTTTGTTATGGC TGG 3' and 5' CCATCATCCTGGCATTTT 3', respectively with a size of PCR product 477 bp and melting temperature (T_m) of 54.5°C (Wang *et al.,* 2008). The forward and reverse primer sequences for exon2 were 5'AGGTTGACTGGTCTCTTTG 3' and 5' ACAGTGGTAGAACTCATGC 3', respectively, with PCR product of 316 bp and T_m of 61.5°C (Wang *et al.,* 2007).

The RFLP analysis of PCR product was carried out by using *Hae*III (5`GG↓CC3`/ 3`CC↑GG5) and *Dpn*I (5`Gm6A↓TC3`/3`CT↑m6AG5`) restriction enzymes for exon1 and *HinfI (*5`G↓ANTC3`/3`C↑TNAG5) and *TruI (*5`T↓TAA3`/3`AAT↑T5`) enzymes for exon2. The reaction was carried out in a 20 µL volume containing 10 µL PCR product, 6.5 µL nuclease free water, 2 µL 10x buffer and 1.5 µL restriction enzymes. The reactions were incubated at 37°C for 14 hr and then analyzed in 1 or 2% agarose gel (Mitra*et al.,* 2011).The data was statistically analyzed by one-way ANOVA and the significance of difference between means was done by Post-hoc test using SPSS software (2012). The genotypes were directly obtained by counting the bands appearing in the agarose gel and their frequency was calculated as the number of animals in a particular group having a particular genotype / total number of animals screened. Association of TLR4 gene variant within affected and non-affected mastitic cases was estimated using chi square (χ^2) test (SPSS software, 2012).

RESULTS AND DISCUSSION

Mastitis screening

A total of 60 animals were selected in normal, subclinical and clinical mastitis group from both Jersey and HF crossbred cattle by clinical examination, CMT and milk EC (Table 1). CMT score of normal group ranged between 0 and trace, subclinical mastitis between 1 and 2, clinical mastitis was 3 and above in both the breeds. A high CMT score was observed for clinical mastitis than subclinical mastitis or normal milk which is in line with Norberg *et al*. (2004). EC values in Jersey cattle in normal, subclinical and clinical groups were 4.048 ± 0.288, 5.425 ± 0.147 and 6.971 ± 0.166, respectively. InHF crossbred cattle the mean of 3 groups were 4.611 ± 0.264, 5.551 ± 0.158 and 7.061 ± 0.243, respectively. In present study, the EC of normal milk from Jersey and HF crossbred was within the range reported by Norberg *et al*. (2004). EC was elevated in animals suffering from mastitis than the normal cattle. This may be attributed to the increase in ionic concentration following the destruction of tight junctions and the active ion pump by bacterial LPS, resulting in effusion of extracellular Na^+ and Cl^- into alveolar lumen of udder (Kitchen *et al.,* 1980). Interestingly, we observed that the EC of normal milk in HF cross bred cattle was higher

Table 1: Performance of normal and mastitic crossbred cattle in screening tests

Breed	Group (n = 10)	CMT score	EC (mS cm^{-1})
Jersey	Normal	0-trace	4.048±0.288*
	Subclinical	1-2	5.425±0.147*
	Clinical	3 or above	6.971±0.166*
HF	Normal	0-trace	4.611 ± 0.264*
	Subclinical	1-2	5.551 ± 0.158*
	Clinical	3 or above	7.061 ± 0.243*

*represents statistically significant figures

than the normal EC of Jersey crossbred. This observation is in line with the observation of Hammann and Zecconi (1998) who recorded difference in EC between breeds and implied that HF milk has higher ionic concentration than Jersey milk.

Mastitis and TLR4 gene polymorphism in Jersey and HF crossbred cattle

In present study, we screened exon1 and exon2 of TLR4 gene in Jersey and HF crossbreeds for polymorphisms by PCR-RFLP. In Jersey, *Hae*III digestion of exon1 generated two fragments of 69 and 408 bp and three genotypes (AA: 69 & 408 bp; Aa: 69, 408 & 477 bp and aa: 477 bp) (Fig. 1). The genotype frequency revealed that in normal non-mastitic cattle 80% were heterozygous and 10% homozygous for both 'A' and 'a' allele at exon1 locus (Table 2). In subclinical mastitis group, 60% animals were homozygous for 'A', 30% heterozygous and 10% homozygous for 'a' allele. In clinical mastitis group, 10% were homozygous for allele "A", 20% heterozygous and 70% homozygous for allele 'a'. The TLR4 exon1 of Jersey cross breed was not digested by *Dpn*I enzyme.

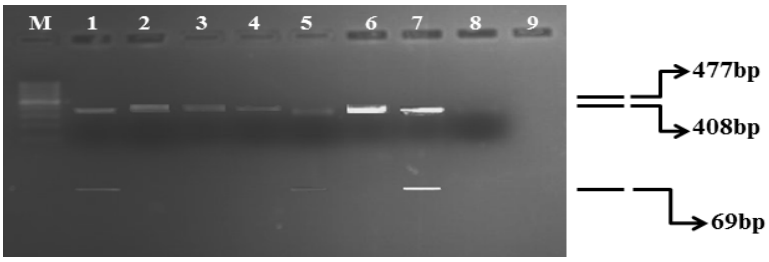

Fig. 1: Polymorphism of TLR4 exon1 in Jersey crossbred cattle (digested with HaeIII enzyme). Lane M–100 bp marker: Lanes 3, 4, 5, 8 & 9 - homozygous mutant (aa); Lanes 1 & 7 - homozygous normal (AA); Lanes 2 & 6 - heterozygous (Aa)

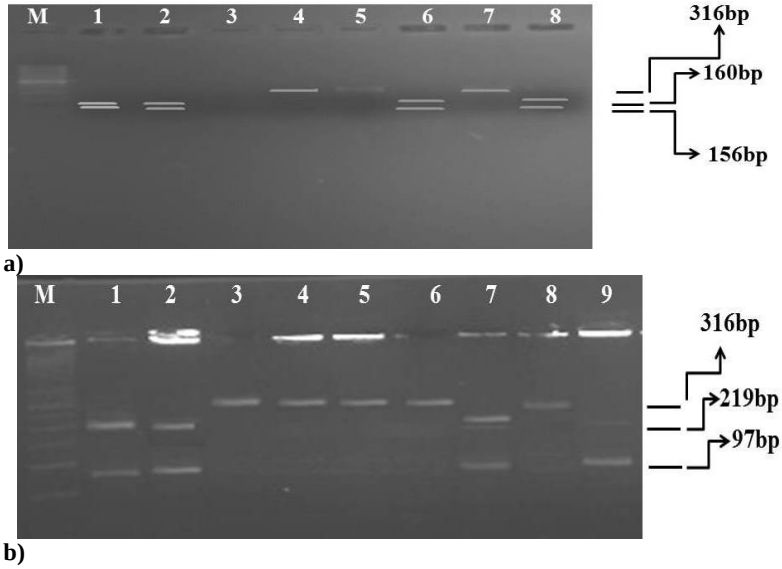

Fig. 2: Polymorphism of TLR4 exon2 in Jersey crossbred cattle digested with a) *HinfI* [Fig 2a] & b) *TruI* [Fig 2b]; In Fig. 2a: Lane M - 100 bp marker; Lanes 1, 2, 3, 6 & 8 - homozygous normal (AA); Lanes 4 & 7 - homozygous mutant (aa); Lanes 5 - heterozygous (Aa). In Fig. 2b: Lane M - 50 bp marker; Lanes 3, 4, 5, 6 & 8 - homozygous mutant (aa); Lanes 1, 2, 7 & 9 - homozygous normal (AA)

Digestion of exon2 of TLR4 gene from Jersey crossbred cattle by *Hinf*I generated 2 fragments (160 and 156 bp) and 3 genotypes (AA: 160 and 156 bp; Aa: 160, 156 and 316 bp and aa: 316 bp) [Fig. 2a]. The digestion of exon2 by *TruI* generated 2 fragments (219 and 97 bp) and 2 genotypes (AA: 219 and 97 bp and aa: 316 bp) [Fig. 2b]. The heterozygous genotype (Aa) at *Hinf*I site was represented by 80, 50 and 20% cattle in normal, subclinical and clinical groups, respectively (Table 2). The genotype 'AA' for *Hinf*I site was represented by 10, 20 and 20% and genotype 'aa' was represented by 10, 30 and 60% cattle in normal, subclinical and clinical groups, respectively. In contrast, for *Tru*I site 60% normal cattle were homozygous for 'A' allele and 40% were homozygous for allele 'a'. In subclinical mastitis group, 30 and 70% were homozygous for allele 'A' and 'a', respectively. In clinical mastitis cases, 40 and 60% cases were homozygous for allele 'A' and 'a' respectively.

In HF crossbred cattle, RFLP analysis of exon1 and exon2 of TLR4 generated identical fragment and the genotypes to that of Jersey cross bred cattle (Fig. 3, 4ab). *Hae*III digestion of

exon1 revealed that the heterozygous genotype (Aa) was represented by 90, 20 and 10% animals in normal, subclinical and clinical mastitis groups, respectively. The genotype 'AA' was represented by 10% in each group and genotype 'aa' by 0, 70 and 80% in normal, subclinical and clinical mastitis groups, respectively (Table 2). Similar to Jersey crossbred cattle, the *Dpn*I enzyme did not generate any genotype in exon1 of HF crossbred cattle. The RFLP analysis of exon2 with *Hinf*I revealed that the genotype distribution in normal group was 90% (Aa), 10% (AA) and 0% (aa). In subclinical group, the genotype distribution was 20 % (Aa), 20% (AA) and 60% (aa) and in clinical group the distribution was 10% (Aa), 30% (AA) and 60% (aa) (Table 2) (Fig 4a).RFLP analysis with *TruI* revealed that 60% animals were homozygous for "A" allele and 40% were homozygous for allele 'a' in normal group. In subclinical mastitis group 70 and 30% were homozygous for allele 'A' and 'a', respectively, whereas in clinical group, 80 and 20% were homozygous for allele 'A' and 'a', respectively (Fig. 4b).

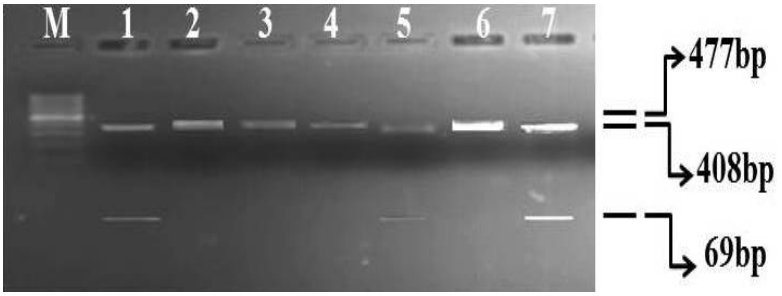

Fig. 3: Polymorphism of TLR4 exon1 in HF crossbred cattle. (digested with *HaeIII* enzyme). Lane M – 100 bp marker; Lanes 2, 3, 4 & 6 – homozygous mutant (aa); Lanes 1, 5 & 7 - heterozygous (Aa).

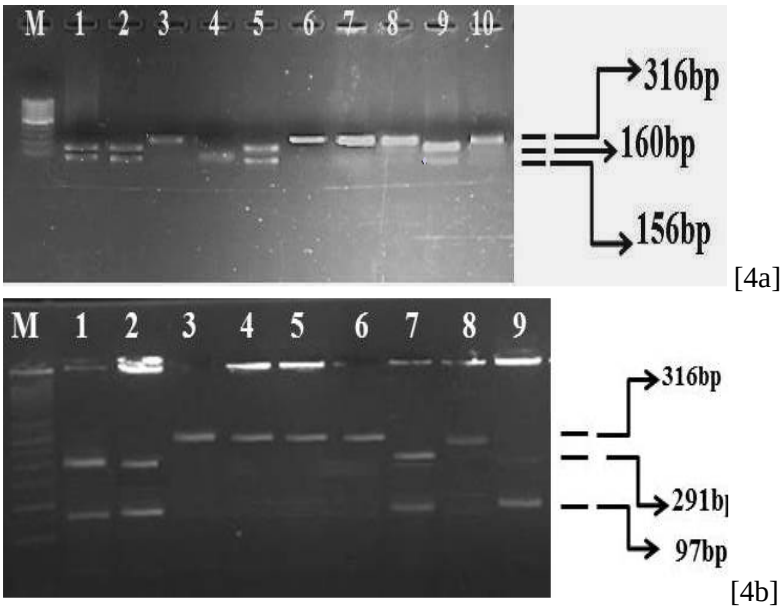

Fig. 4: Polymorphism of TLR4 exon2 in HF crossbred cattle (digested with *HinfI* (Fig 4a) &*TruI* (Fig 4b). Fig. 4a): Lane M – 100 bp marker; Lane 1, 2, 5 & 9 - homozygous normal (AA); Lanes 3, 6, 7 & 8 - homozygous mutant (aa); Lanes 4 & 10 - heterozygous (Aa). Fig. 4b): Lane M – 50 bp marker; Lanes 4, 5, 6, 7 & 9 - homozygous mutant (aa); Lanes 1, 2, 3 & - 8 homozygous normal (AA)

The polymorphism obtained by RFLP analysis in exon1 and exon2 of TLR4 gene were associated with the susceptibility or resistance to mastitis using χ^2 test (Table 2). In Jersey crossbred cattle, the analysis revealed no significant association of the polymorphism in exon1 or exon2 of TLR4 gene with the susceptibility to mastitis. In HF crossbred cattle, the analysis showed a significant association of polymorphism in exon1 at *Hae*III site and in exon2 at *Hinf*I site with the susceptibility to mastitis. The HF crossbred animal with genotype 'aa' in both exon1 and exon2 were significantly susceptible to the mastitis.

Mastitis is a disease of paramount importance with high incidence of clinical and subclinical disease in dairy cattle (Kenar*et al.*, 2012; Islam *et al.*, 2014). TLR4 genes have been reported to be important for LPS recognition of pathogens and subsequent elicitation of immunological responses (Poltorak*et et al.*, 1998; Swiderek *et al.*, 2006;

Table 2: Genotypic and gene frequency of exon1 and exon2 of TLR4 gene and their association with mastitis in Jersey and HF crossbred cattle

Breed	Exon	Enzyme	Group	AA	Aa	Aa	χ2	p value	A	A	χ2	P value
Jersey	Exon1	*Hae*III	Normal	0.10	0.80	0.10	9.197	0.056	0.50	0.50	4.775	0.091.
			Subclinical	0.10	0.30	0.60			0.25	0.75		
			Clinical	0.10	0.20	0.70			0.20	0.80		
	Exon2	*Hinf*I	Normal	0.10	0.80	0.10	7.800	0.099	0.50	0.50	1.786	0.410.
			Subclinical	0.20	0.50	0.30			0.45	0.55		
			Clinical	0.20	0.20	0.60			0.30	0.70		
		*Tru*I	Normal	0.60	0.00	0.40	1.900	0.380	0.60	0.40	3.800	0.140
			Subclinical	0.30	0.00	0.70			0.30	0.70		
			Clinical	0.40	0.00	0.60			0.40	0.60		
HF	Exon1	*Hae*III	Normal	0.10	0.90	0.00	17.100	0.001*	0.55	0.45	9.040	0.010*.
			Subclinical	0.10	0.20	0.70			0.20	0.80		
			Clinical	0.10	0.10	0.80			0.15	0.85		
	Exon2	*Hinf*I	Normal	0.10	0.90	0.00	16.555	0.002*	0.55	0.45	2.912	0.232
			Subclinical	0.20	0.20	0.60			0.30	0.70		
			Clinical	0.30	0.10	0.60			0.35	0.65		
		*Tru*I	Normal	0.60	0.00	0.40	0.90	0.620	0.60	0.40	1.900	0.380
			Subclinical	0.70	0.00	0.30			0.70	0.30		
			Clinical	0.80	0.00	0.20			0.80	0.20		

*represent statistically significant figures

Sharma *et al.,* 2006). Earlier studies have reported that mutation in TLR4 gene increases the susceptibility of cattle to mastitis (Sentitula *et al.,* 2012). Wang *et al.* (2007) have found 36 SNPs in TLR4 gene across 13 different cattle breeds in China. White *et al*. (2003) have identified several polymorphisms in bovine TLR4 gene, many of which reportedly affect somatic cell count during mastitis. The present study revealed that the polymorphism is prevalent in TLR4 gene and are significantly associated with mastitis in HF cross-bred cattle. Of importance, this study revealed no site for *Dpn*I in exon1 of both Jersey and HF crossbred cattle. Although this enzyme was selected for RFLP analysis based on restriction analysis of sequence of TLR4 gene of Jersey and HF cattle that showed at least one site in exon1. This finding implies that crossbreeding of Jersey and HF with indigenous Kashmir cattle might have resulted in the loss of *Dpn*I site in exon1. The study showed a significant association between mastitis and two loci of TLR4 gene in HF crossbred cattle *viz.*, *Hae*III site in exon1 and *Hinf*I site in exon2. However, Jersey crossbred cattle showed no association between mastitis and these two loci of TLR4 gene. Our findings are supported by Wang *et al.* (2007) and Noori *et al.* (2013) who found several SNPs in TLR4 gene significantly associated with mastitis in Chinese HF crossbred and Iranian HF crossbred cattle, respectively. In a study on Chinese Simental, HF and Sahne cattle breeds, SNPs at *Hinf*I in TLR4 gene have been found to be associated with mastitis (Wang *et al.,* 2008). In conclusion, the present study revealed significant association between bovine mastitis and polymorphism in TLR4 gene in HF crossbred which suggests its important role in mastitis pathogenesis in local HF crossbred cattle of Kashmir.

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