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Full Length Article

Identification of Polymorphism in Bovine Tumor Necrosis Factor Alpha and Toll-like Receptor 4 Genes and its Association with Mastitis in Sahiwal Cows

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Abstract

High prevalence of udder infection is one of the major problems faced by dairy farmers. Despite considerable technological advancement, udder inflammation (mastitis) is still widespread, inflicting high production losses to the dairy industry of Pakistan. It directly affects the milk yield, quality of milk and farm economics. Identification of sequence markers that are directly associated with resistance to mastitis might be important animal selection traits for the cost associated with the prevention and treatment of disease. The present study aimed at identifying polymorphism in tumor necrosis factor alpha (TNF-α) and toll like-receptor 4 (TLR4) genes of 50 Pakistani Sahiwal cows (clinical n= 20, subclinical n= 20, normal n= 10). Novel polymorphic sites (4 in TNF-α and 8 in TLR4)were identified within the studied parts of these genes and can serve as genetic markers for identification of mastitis resistant and susceptible animals. This is the first report of identification of molecular markers for screening of mastitis resistant and susceptible Sahiwal cows using immunity genes. © 2018 Friends Science Publishers

Keywords: Mastitis; TNF-α; TLR4; Immunity; Sahiwal cows

Introduction

Mastitis is inflammation of mammary glands and potentially a highly contagious devastating disease that can cause severe economic losses in affected farms (Fourichon *et al*., 2005; Kenyanjui and Sheikh-Ali, 2011). The severity of mastitis is closely tied to the genetic makeup of the animal and is greatly influenced by the invading pathogens and subsequent inflammatory response depending upon recruitment of immune cells. In spite of a considerable technological advancement, still it is very difficult to get around the mastitis problem because several genetic and environmental factors are involved in its etiology (Bradley, 2002; Togashi and Lin, 2010) Inherited resistance against mastitis is a polygenic complex trait. Among these, the polymorphism in immunity genes could potentially be associated with mastitis resistance and susceptibility and could be used as a tool in the selection of mastitis resistant animals (Li *et al.*, 2009). Due to genetic amenability and stability, the polymorphism based molecular markers have received great attention. Identification of sequence markers that are directly associated with immunity of mastitis might be important selection traits for reducing the cost incurred on the prevention and treatment of this disease (Togashi and Lin, 2010). Among these immunity genes, the tumor necrosis factor alpha (TNF-α) and toll-like receptor 4 (TLR4) have been proved to play key role in mastitis resistance and susceptibility. These genes display many biological functions related to the host immune system. Some studies have demonstrated that both the genes are highly polymorphic and few specific variations are directly associated with onset of mastitis (White *et al*., 2003; Ogorevc *et al*., 2009).

TNF-α is the main pro-inflammatory adipokine that is part of systematic immune defense and stimulates the specific immune system. This gene is responsible for proliferation, differentiation and activity of B lymphocytes and Natural killer cells. It also induces the production and release of many cytokines (Wojdak-Maksymiec *et al*., 2013) and also enhances the phagocytic and chemotactic effects of immune response (Bannerman, 2009; Moyes *et al*., 2009). Toll-like receptor 4 (TLR4) is a major recognition receptor. TLR4 recognizes a great variety of pattern associated molecular pathogens (PAMPs), specifically the pathogen ligands and consequently contributes directly to the innate and adaptive inflammatory response (Wang *et al*., 2007).

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The present study was designed to investigate the genetic polymorphisms in exon 4 and exon 3 of TNF-αTLR4 gene, respectively of Sahiwal cows.

The present study proposed TNF-α and TLR4 genes as major candidate genes related to mastitis resistance or susceptibility in Sahiwal cattle and can be used in marker assisted selection program to promote the inheritance of resistance trait in this dairy breed. This is the first report of single nucleotide polymorphism (SNPs) identification of TNF-α and TLR4 genes of the Pakistani Sahiwal cattle and their association with bovine mastitis.

Materials and Methods

To explore the polymorphisms within TNF-α and TLR4 genes, blood samples were collected from a total of 50 Sahiwal cows (clinical mastitis n=20; subclinical mastitis n=20; non mastitis n=10). The samples were named as clinically mastitic (CM1 thru CM20), sub-clinically mastitic (ScM1 thru ScM20) and normal Sahiwal (NS1 thru NS20). All animals were kept under similar managemental conditions. For the detection of sub-clinical cases, Surf Field Mastitis Test (Muhammad *et al*., 1995) was performed as the animal side test. To this end, quarter foremilk samples were mixed with an equal quantity of 3% solution of household detergent (Surf Excel, Unilever, Pakistan) in the four receptacles of the Surf test paddle. The mixture was gently rotated for about 20 sec and then examined for gel formation. The total DNA was isolated from blood samples by commercially available Genomic DNA isolation kit™ (Fermentas Co. USA).

For amplification, two sets of primers were designed using available *B. taurus* TNF-α andTLR4 gene sequences (NCBI GenBank; Accession no. AC_000180.1 and AY634630.1, respectively) (Table 1).

Amplification was carried out with following temperature profiles: initial denaturation-95^{o}C for 5 min, denaturation- 94^{o}C for 30 sec, annealing -54^{o}C for 30 sec, extension -72^{o}C for 1 min, repeat step 1-3 for 30 cycles, final extension -72^{o}C for 10 min. Purified amplicons were subjected to sequencing using BigDye terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, CA, USA). Sequence data were edited manually using Chromas software Ver. 1.45 followed by sequence alignments using NCBI BLAST freeware.

Results

In the presents study, the TNF-α and TLR4 genes of Pakistani Sahiwal cattle were sequenced. Comparative analysis of these two gene sequences with reference sequence revealed SNPs at different positions. In TNF-α gene sequence of clinical and sub clinical mastitic cows, three transition SNPs were identified at location C111T (C/T), C209T (C/T) and A308G (A/G) in exon 4 (Table 2).

A comparative analysis of TLR4 gene sequence of 555bp of clinically and subclinically mastitic Sahiwal cows revealed 8 SNPs (transition= 06 and transversion= 02) in sequence at multiple locations. Eight SNPs found in different positions of the gene; C10477T, G10689A, T10727C, C10738T, A10739G, A10768G showed transition polymorphism and A/T (10554), C/G (10814) showed transversion polymorphism (Table 3).

Discussion

Mastitis is one of the most economically devastating and deleterious diseases of dairy cattle that presents a great global challenge to the dairy industry. Development of new strategies to combat this problem requires an understanding of the molecular basis of resistance and susceptibility towards mastitis (Angulo *et al*., 2004). Various approaches have been employed in attempts to improve understanding of mastitis resistance and susceptibility, and to identify phenotypic and molecular markers for control of this disease (Afzal, 2010). In the present study, the TNF-α and TLR4 genes of indigenous Sahiwal cows with clinical and without mastitis signs (subclinical) were examined.

Polymorphisms were determined in the TNF-α and TLR4 genes of clinical and sub clinical mastitis samples of Sahiwal cows. These candidate genes are associated with the immunity system and responsible for initial recognition of invading microorganisms. The polymorphisms identified were the SNP TNF-α 111 (C >T), SNP TNF-α 209 (C >T) and SNP TNF-α 308 (A>G) which were located in the exon 4 of this gene. These polymorphisms were also predominant in the Chinese Holstein cow population studied by Shirasuna *et al*. (2011) and Wojdak-Maksymiec (2013). All these transitions were present in both clinical and subclinical cases. These findings suggested that all these SNPs affect immune function of the host and are associated with the risk of mastitis. The present marker based study may potentially enhance the niche area of mastitis research and thereby help in devising a suitable resistance trait breeding approach to control this economically important disease (Detilleux *et al*., 1995). By advocating the mastitis resistant genotype in breeding programs, the natural immune response of the animal can be improved to better fight against invading pathogens. This study may contribute to a better understanding of the immune system to impart resistance against mastitis.

Conclusion

None of these SNPs were associated with differentiation of clinical and subclinical cases. As far as could be ascertained from the available literature, the present study represents the first attempt to measure the SNP based disease resistant/susceptible markers in Sahiwal cows. In view of the preliminary nature of the study, additional work along

Table 1: Details of the primers used to investigate the polymorphic identification in TNF-α gene and exon 3 of TLR4 genes

Primer Name	5'-3' Sequence	Tm (°C)	Product size (bp)
TNF-F	ACTGACAGGGTCGCACTGAT	57.5	
TNF-R	GATCGCCTCAGTGCTGAGAT	57.2	370
TLR4-F	CGCCATGTACCAAGCATTGT	61.2	
TLR4-R	GGGTTGATAGAGAAGACGTGG	61.2	555

Table 2: SNPs identified in TNF-α gene sequence of clinical mastitis sample of Sahiwal cows

Position	Reference	SNPs	Type of mastitis	Results
111	C	T	Clinical & subclinical	Transition
209	C	T	Clinical & subclinical	Transition
308	A	G	Clinical & subclinical	Transition

Table 3: SNPs identified in TLR4 gene sequence of Sahiwal cows affected with clinical and subclinical mastitis

Position	Reference	SNPs	Type of mastitis	Results
10477	C	T	Clinical & subclinical	Transition
10554	A	T	Clinical & subclinical	Transversion
10689	G	A	Clinical & subclinical	Transition
10727	T	C	Clinical & subclinical	Transition
10738	C	T	Clinical & subclinical	Transition
10739	A	G	Clinical & subclinical	Transition
10768	A	G	Clinical & subclinical	Transition
10814	C	G	Clinical & subclinical	Transversion

these lines is clearly warranted before recommending incorporation of these markers in the national breeding policy of disease resistant Pakistani Sahiwal cows.

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