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Short Communication

Genetic Association of Bovine *TNF-α* Gene Polymorphism with Clinical and Sub-clinical Mastitis in Sahiwal Cows

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HS, MSH and AIA performed the research. HS prepared the first draft. SF and HUR conceived the idea. ARW revised the manuscript and analyzed findings.

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ABSTRACT

One major problem of dairy cattle industry is the high prevalence of mammary gland infection. Despite significant technological developments in animal husbandry, udder infection is still widespread; particularly in large herds that causes high production losses to the dairy industry of Pakistan. Identification of molecular marker sequences that are directly related to immunity against mastitis might be helpful for animal selection trait and prevention of this disease. The aim of the present study was to assess the effect of polymorphism in bovine tumor necrosis factor alpha (*TNF-α*) gene on immune function and its role in mastitis susceptibility in Pakistani Sahiwal cows. In this study, a total of 150 Sahiwal cows were selected suffering from clinical (n=50), subclinical (n=50) mastitis along with non-mastitic (n=50) cows from different dairy farms of Punjab. *TNF-α* gene was amplified with specific primer and sequenced to get the full-length sequence of this gene. A total of nine changes including transition (n=6) and transversion (n=3) were found at the different position of this gene. Due to these changes, the amino acid is changed that leads to significant change in the folding of 3D protein structure of clinical sample, while in subclinical samples, showing the same variation in overall 3D protein structure analysis of *TNF-α* gene. The association between polymorphism identified within the *TNF-α* gene with mastitis reported in this study revealed that SNPs has potential to serve as a molecular marker for screening of mastitis resistant and susceptible Sahiwal cows.

One of the most prevalent disease of the dairy industry is mastitis that playing a devastating role for economic loss in the affected farms (Aqib *et al*., 2019b). Economic loss includes poor milk quality, low milk production, cost of treatment, labor charges, premature culling and increased risk of other diseases (Fourichon *et al*., 2005; Ali *et al*., 2011). Despite considerable technological advancement, it is still very challenging to control mastitis because several factors (environmental and genetic) are involved in etiology of mastitis (Carvajal *et al*., 2013; Aqib *et al*., 2019a; Mahboob *et al*., 2018). Mastitis resistance and susceptibility is a complex trait influenced by genetic variation. Among these variations, the polymorphism in immunity genes associated with mastitis are primary key factors in defensive mechanism of mammary gland (Rupp and Boichard, 2003; Ibeagha-Awemu *et al*., 2008).

Dairy cattle are more susceptible to mastitis because of poor hygiene conditions and weak mammary gland defense mechanism (Sordillo and Streicher, 2002). Various cellular and soluble immune components are involved in protecting the mammary gland from infectious diseases. The mammary gland tissue is protected by innate and acquired immune system (Mesquita *et al*., 2012). A vast variety of cytokines associated with acquired immune system, among which tumor necrosis factor alpha (*TNF-α*) gene is a candidate factor that has been proven to play important role in mastitis susceptibility and resistance.

TNF-α is main pro-inflammatory adipokine that is a member of cytokine group of systematic immune

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defense. It is involved in proliferation, differentiation, and activation of many immune system cells including B lymphocytes, NK (natural killer) along with the stimulation and release of other cytokines (Wojdak-Maksymiec and Mikolajczyk, 2012). It is 17 KDa molecule consist of 212 amino acids arranged in stable homotrimers and composed of two β-pleated sheets and β-strands, joined together antiparallel (Tang *et al*., 1996). *TNF-α* gene has BTA23q22 chromosomal position, having four exons and three introns (Bannerman, 2009; Moyes *et al*., 2009). This gene is involved in different biological functions of host defense system, hence polymorphism in the *TNF-α* gene could be used as a tool for selection of mastitis resistant animal selection (Parameswaran and Patial, 2010).

The present study is designed to investigate the polymorphism in bovine *TNF-α* gene and its effect on change in an amino acid sequence leading to change in the 3D structure of a protein in Pakistani Sahiwal dairy cattle. A subordinate objective of the study is to find out the *TNF-α* gene-based molecular marker to differentiate between mastitis susceptible and resistant Sahiwal cows to promote the inheritance of resistance trait.

Materials and methods

For the identification of polymorphism within *TNF-α* gene, a total of 150 Sahiwal cows (clinical mastitis n= 50; subclinical mastitis n=50; non-mastitis n=50) were selected. For subclinical mastitis detection, Surf Field mastitis test (Muhammad *et al*., 1995) was performed at the animal site. For this purpose, 3% solution of household detergent (Lever Brothers, Surf Excel, and Arial) was mixed with quarter foremilk sample in equal volume and was swirled for 15-20 seconds and looked for thickening of the mixture (i.e. gel formation). About 5-10 mL blood sample was collected from jugular vein aseptically in blood vacutainer tubes. Organic DNA extraction method was used for DNA extraction from blood samples (Sambrook *et al*., 2001).

For amplification of *TNF- α* gene, six sets of primer were designed using already reported sequence of *B. taurus* from NCBI (NCBI GenBank; Accession no. AC_ 000180.1) (Table I). Optimization of primers was carried out with following temperature profile; initial denaturation-95°C for 5 min, denaturation-94°C for 30 s, annealing of TNF1 at 56°C, TNF2 at 60°C, TNF3 at 55°C, TNF4 at 52°C, TNF5 at 56°C and TNF6 at 58°C for 30 s, extension-72°C for 60 s, final extension-72°C for 10 min, repeat step 1-3 for 30 cycle.

Purified amplicon was subjected for commercial sequencing. The sequencing result was analyzed manually using BioEdit software followed by sequence alignment through NCBI BLAST. Protein analysis was done using ExPasy translate tool along with the development of 3D structure using PAYMOl software.

Results

In this present study, the *TNF- α* gene of Pakistani Sahiwal cow was sequenced for identification of polymorphism. Comparative sequence analysis with reference sequence revealed SNPs at different position of the gene. In *TNF- α* gene sequence of clinical and subclinical mastitic cows, six transition SNPs were identified at location 268 (A>G), 383 (G>A), 750 (C>T), 2075 (A>G), 2444 (T>C), 2511(C>T). Three changes were identified at position 538 (G>C), 1139 (T>G) and 2512 (T>G) that show transversion polymorphism (Table II). The protein analysis shows missense mutation in amino acid sequence i.e., Leucine is changed with Tryptophan at 215 (Fig.1).

Table I.- Details of primers used to investigate the polymorphism within *TNF- α* gene

Primer Name	5'-3' Sequence	GC (%)	Product size (bp)
TNF1 F	CTTCCCTTTCTCCAGCTCCT	55	
TNF1 R	GAGACAGGAGAGCCTTGTGG	60	692
TNF2 F	CCACAAGGCTCTCCTGTCTC	60	
TNF2 R	TGCTTACTCATTCGTCCAACA	43	484
TNF3 F	TGTTGGACGAATGAGTAAGCA	43	
TNF3 R	ACCCTGCACTGTCTCTCTGC	60	690
TNF4 F	CTGGGTCATGAAGAAGCAGAG	53	
TNF4 R	TAGTCCGGCAGGTTGATCTC	55	664
TNF5 F	GAGATCAACCTGCCGGACTA	55	
TNF5 R	GCAAACATAAACAGAGGGAGTTG	44	524
TNF6 F	AACTCTCCCTTCCTGCCAAT	50	
TNF6 R	AAGAGGCCCATGCAGAATTA	45	480

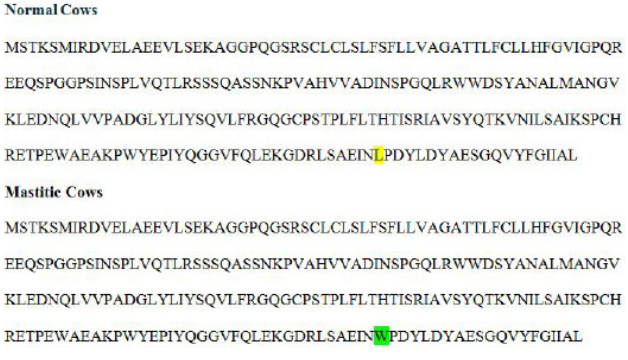

Fig. 1. Deduced amino acid sequence of *TNF-α* gene of normal and mastitic Sahiwal cows.

Table II. SNPs identified in *TNF- α* gene sequence in clinical & subclinical mastitic Sahiwal cows.

Position	Reference	SNPs	Region	Type of mastitis	Results
268	A	G	Intron	Clinical & Subclinical	Transition
383	G	A	Intron	Clinical & Subclinical	Transition
538	G	C	Intron	Clinical & Subclinical	Transversion
750	C	T	Exon	Clinical & Subclinical	Transition
1139	T	G	Intron	Clinical & Subclinical	Transversion
2075	A	G	Intron	Clinical & Subclinical	Transition
2444	T	C	Exon	Clinical & Subclinical	Transition
2511	C	T	Exon	Clinical & Subclinical	Transition
2512	T	G	Exon	Clinical & Subclinical	Transversion

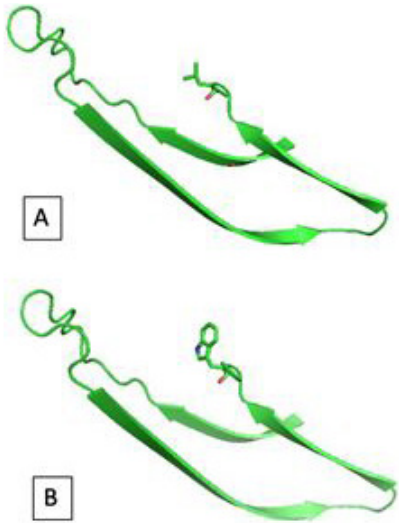

Fig. 2. 3D modeling for the peptide of *TNF-α* gene of normal (A) and mastitic (B)cows

Discussion

SNPs were identified in *TNF- α* gene in both clinical and subclinical cases but not in non-mastitic samples so, this candidate gene are associated with cow immune response against invading microorganism. A total of nine changes including transition (n=6) and transversion (n=3) were found at different positions (exon and intron) of this gene. The polymorphism at position 111 (C/T), 209 (C/T) and 308 (A/G) in exon 4 of *TNF-α* were reported by Shirasuna (2011) and Maksymiec (2013) in Holstein-Friesian cows and Firyal (2018) in Sahiwal cows (Shirasuna *et al*., 2010; Wojdak-Maksymiec *et al*., 2013; Firyal *et al*., 2018) but the other SNPs are not reported.

The protein analysis performed indicates that there is a change in amino acid from Leucine to tryptophan at amino acid position 215. These mutational changes in polar groups produce a change in the overall 3D protein structure of *TNF- α* gene (Fig. 2).

All these changes were found in both clinical and subclinical mastitis samples of Sahiwal cows at different intronic and exonic region. A comparison of the *TNF-α* gene in various type of mastitis in the present study indicates variations within the *TNF-α* gene that is responsible for mastitis. So, *TNF-α* is a potential candidate gene for the screening of the mastitis susceptible and resistant dairy cows.

Conclusion

The present investigation demonstrates the presence of nucleotide changes at various positions in bovine *TNF- α* gene that leads to change in amino acid sequence. Our result suggests that these SNPs may be used as a potential genetic marker for screening of mastitis resistant and susceptible Sahiwal cows. This can be useful in the selective breeding of cattle for an enhanced immune response as a tool to improve inherent animal health.

Statement of conflict of interest

The authors declare no conflict of interest.

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