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SHORT COMMUNICATION

Polymorphism Analysis of Exon 2, 5 and 10 of Bovine Lactoferrin Gene and its Association with Mastitis in Sahiwal Cows

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ABSTRACT

The present study aimed at identifying single-nucleotide polymorphism (SNP) in coding region of bovine lactoferrin gene in Sahiwal cows to establish the baseline of polymorphism. A total of 30 animals from Sahiwal breed were screened for three loci of lactoferrin gene; LtfE2, LtfE5 and LtfE10. Total of 6 SNPs with monomorphic pattern of distribution were identified in three studied loci. These polymorphisms indicated the existence of amino acids variants in the Sahiwal cows affected with mastitis. These SNPs could serve as potential markers for association with susceptibility or resistance to mastitis in Sahiwal cows. This lactoferrin has the potential to serve as candidate gene for the selection of mastitis resistance. Sahiwal cows in the animal breeding program to improve the animal health.

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INTRODUCTION

Sahiwal cow plays a major role in the dairy industry of Pakistan. Although, these cows are said to be relatively less susceptible to some of the tropical diseases, infections of mammary gland (mastitis) continues to cause huge economic losses to the dairy farmers (Khan and Khan, 2006). Almost 20% cows and buffaloes are affected with mastitis, due to which not only milk production decreased but also the quality of milk affected. Pakistan is ranked 3rd in the world in relation to milk production but on the other hand, the losses due to mastitis only in Punjab are nearly to Rs. 240 million per year (Afzal, 2010). Lactoferrin (*LF*), an iron-binding glycoprotein is well known to act as a general antibacterial molecule (Adlerova *et al*., 2008). Lactoferrin is a ferric ion (Fe3 þ) binding glycoprotein found mostly in milk and is a member of transferrin family. If the amount of *LF* is higher in milk, it reduces the chances of mastitis. In the preface analysis of *LF*, it was thought that it stops the growth of microorganisms due to its bacteriostatic ability and stop ferric ion demands of microorganisms. It plays an exceptional role in the innate defense system of mammary gland of dairy animals. Further *LF* has been found to reduce the invasion of *Staphylococcus aureus* in mammary epithelial cells. Thus, variations in *LF* concentration in normal lactating and mastitis cows and its antibacterial activity make it a unique candidate marker for genetic selection towards reduced mastitis susceptibility of dairy animals. The gene encoding for bovine *LF* is organized into 17 exons and polymorphisms within bovine *LF* gene have been reported to be associated with susceptibility/ resistance to mastitis (Schwerin *et al.*, 1994). To date, the polymorphisms in the *LF* gene have not been described in Sahiwal cows, so this is the first report of molecular understanding and role of *LF* gene in mastitis. Such studies are important in the quest to develop strategies to impart and promote resistant genotype against mastitis in the population. In the present study, we described SNPs through polymerase chain reaction and DNA sequencing within three exons (LtfE2, LtfE5 and LtfE10) of bovine LF gene.

MATERIALS AND METHODS

To explore the variations within bovine *LF* gene, blood samples were collected from a total of 30 animals (Clinical mastitis n=20; Subclinical mastitis n=20; Normal n=10) belonging to well-recognized Sahiwal breed of Pakistan. All animals were kept under similar environmental conditions. For the detection of sub-clinical cases, Surf field mastitis test (Muhammad *et al*., 1995) was performed as the animal side test. Blood was collected from jugular vein of selected animals into EDTA containing vacutainer tubes, and DNA extraction was performed from whole blood following organic extraction method. A total of three sets of primers were designed using PRIMER3 software for PCR (Table 1). Total PCR reaction volume was 25 µl containing DNA (50 ng/ µl), ammonium persulphate buffer

(X10), MgCl2 (2.5 mM), dNTPs (25 mM), 10 pmol of forward and reverse primer (Macrogen Inc., Applied Biosystems, USA), 0.3 U Taq DNA polymerase (Fermentas; Glen Burnie, Maryland) and distilled water. PCR products were purified using absolute ethanol and purified amplicons were subjected for sequencing. Sequence alignments were performed with NCBI BLAST and the constructed haplotypes of different exons were conceptually translated to amino acid sequences using Expasy translate tool software.

Table 1: Primers used in study

Locus	Oligonucleotide sequence (5`-3`)	Product size (bp)	Tm (^{0}C)
LtfE2 F	gggacagtgtagaagccctcac	257	56
LtfE2 R	atccagtctggtttccttcagtctggt	257	
LtfE5 F	aaaggctatcaggtgggggtggag	259	54
LtfE5 R	ggccgctggggagaagagga	259	
LtfE10 F	ggcacctgacgtccgttctcttag	209	64
LtfE10 R	gccaggggtactcttctccacct	209	

RESULTS AND DISCUSSION

Mastitis is one of the most economically devastating and limiting factor in the progress of dairy sector of Pakistan (Hussain *et al*., 2013; Izquierdo *et al*., 2017). It is very difficult to comprehend with this disease because numerous environmental and genetic factors are involved in the origin and development of mastitis (Carvajal *et al*., 2013) susceptibility and resistance to mastitis is a complex trait influenced by genetic variation of animal. Among these variations, the polymorphisms in immunity genes are principle key factors in defensive mechanism of mammary gland. For the development of new rational approaches to combat this problem requires an understanding of the molecular basis of this resistance and susceptibility. The present study attempts to highlight the role of *LF* gene as candidate marker for screening of mastitis resistance and susceptibility in Sahiwal cows. As this gene, has very important role in the regulation of defense mechanism of the mammary gland (Adlerowa, 2008).

In the present study, the coding region of LF gene of Pakistani Sahiwal cows with clinical and without mastitis signs were sequenced. Comparative analysis of this gene sequences and the reference sequence revealed SNPs at different locations (Table 2). In LF gene sequence of clinical and sub clinical mastitis cows, total six SNPs were identified in exons 2, 5 and 10. In exon LtfE2 one transversion SNP was identified at location 5343390 (G/T) and one transition SNP was identified at position 5342422 (C/T). In exon LtfE5 two transversion SNPs were identified at location 5343493 (G/T) and 5343480 (T/C). Two transition SNPs were identified at location 5343388 (G/A) and at location 5343385 (A/G) in exon LtfE10. All these six polymorphisms are novel and have not been reported yet in Pakistani Sahiwal cows. These polymorphisms were present in both clinical and subclinical cases. Previously, the same gene was genotyped by Carvajal and his co-workers to find out the specific genotype of animal associated with healthy and disease condition. The results of present study show the significant relationship between the polymorphisms in coding region and onset of both types of mastitis. So, the utility of this gene as a marker for screening of resistant animal against mastitis augment the findings of the previous works conducted by Phuktes *et al*. (2001) and Wang *et al*. (2009). After that protein analysis was done with the help of ExPasy translate tool. Three of these polymorphisms are responsible for 3 amino acid variations. Significant changes detected in LtfE10 exon, on which due to this SNP Glutamic acid was replaced by stop codon and as a result truncated protein formed and in LtfE2 Phenylalanine changed in to cysteine and in LtfE5 leucine changed into proline Theses variations analysis may help in future association studies for susceptibility/ resistance to mastitis in Sahiwal cows. In the present study, none of these 06 SNPs have been verified as a potential genetic marker for *Lf* gene based molecular differentiation of clinical and subclinical cases. As far as could be ascertained from the available literature, the present study represents the first attempt to identify the markers of resistance/ susceptibility to mastitis is Sahiwal cows. In view of the preliminary nature of the study, additional work along these lines is clearly warranted before recommending incorporation of *Lf* gene in the national breeding policy of Pakistani Sahiwal cows.

Conclusions: In our study, the 06 unique SNPs of Lf gene were verified as molecular marker for identification of mastitis resistant and susceptible animal.

Authors contribution: SF and SM identified the problem and carried out the experiment, data analysis and manusripct writeup. MT and SS analysed the data. ARA, MW and GM edited the manusript.

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Table 2: SNPs and amino acid change identified in *LF* gene sequence of Sahiwal cows with mastitis

Sr. No	Exons	Position	Reference	SNPs	Type of mastitis	Results	Amino acid change
1.	LtfE2	5343390	G	T	Clinical & subclinical	Transversion	Phenylalanine to cystine
2.	LtfE2	5342422	C	T	Clinical & subclinical	Transition	No change of amino acid
3.	LtfE5	5343493	G	T	Clinical & subclinical	Transversion	Leucine to proline
4.	LtfE5	5343480	T	C	Clinical & subclinical	Transversion	No change of amino acid
5.	LtfE10	5343388	G	A	Clinical & subclinical	Transition	No change of amino acid
6.	LtfE10	5343385	A	G	Clinical & subclinical	Transition	Glutamic acid to stop codon