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Full Length Article

Genetic Association of Polymorphism and Relative mRNA Expression of Tumor Necrosis Factor-Alpha Gene in Mastitis in Sahiwal Cow

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Abstract

Bovine mastitis is a host response to the microorganisms linked with the host immune system efficiency. Tumor necrosis factor-alpha (*TNF-α*) is a proinflammatory cytokine that plays a significant role in the innate and adaptive immune response. In this study, we characterized the upstream regulatory region and evaluated the relative mRNA expression of *TNF-α* gene of Sahiwal cows. A single nucleotide polymorphism A>G was identified located within a sequence (MT_919286) at the 5´ upstream region. For gene expression, the ΔΔCt was calculated by adjusting the target gene expression for the expression of the housekeeping gene (*GAPDH*) through real-time qPCR. The results revealed that relative mRNA expression of *TNF-α* most explains the change in the unit of ΔCt and would result in a significantly higher expression of *TNF-α* gene in animals with mastitis. The relative mRNA expression of *TNF-α* gene was 35 and 9.53 times higher in animals with clinical and subclinical mastitis respectively, as compared to non-mastitic animals. The effect of the fold change of *TNF-α* and *GAPDH* was also assessed based on response surface methodology via Box Behnken design. The analysis depicted that all parameters had a significant impact on mastitis incidence in Sahiwal cows. This study would hopefully contribute towards a better understanding of the use of *TNF-α* gene marker as an authentic source of identification of severity of bovine mastitis. The findings of study may be helpful for the development of new strategies to control mastitis and preserve the health of dairy animals.

Keywords: Mastitis; Sahiwal cow; *TNF-α*; Gene expression; Polymorphism; Promoter analysis

Introduction

Mastitis is inflammation of mammary glands regardless of the cause. Currently, it is one of the most prevalent and economically important disease of dairy animals (Rehman *et al*. 2017; Cobirka *et al.* 2020). Different avenues of colossal economic losses associated with mastitis include milk discarded after treatment (9%), decreased milk yield (70%), extra labor (4%), and veterinary services cost (7%), and early culling (14%) (Dua 2001). Estimates of mastitis prevalence in cows in different studies ranged from 29.34–78.54% (Ebrahimi *et al*. 2007; Sharma and Maiti 2010) while in dairy buffaloes, prevalence varied from 27.36–70.32%.

According to the presence or absence of clinical signs, there are two forms of mastitis viz. clinical and subclinical. Clinical mastitis is characterized by visible signs of inflammation in the udder (redness and swelling, fever etc) and alterations in the appearance of milk (such as presence of flakes and clots, watery consistency of milk). Subclinical mastitis on the other hand is bereft of visible changes in the udder and in the milk (Muhammad *et al*. 2010; Cobirka *et al*. 2020).

The fundamental principles of mastitis control program currently in vogue worldwide were developed during the 1960s by the National Institute for Research in Dairying (NIRD), UK. Despite 60 years application of this program, the prevalence of mastitis even in developed countries is still unacceptably high and this has spurred interest into additional mastitis control strategies notably breeding animals for mastitis resistance (Sender *et al*. 2013). A tremendous volume of research has been done on genetic

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basis of mastitis resistance in Holstein-Friesian, Jersey and some other breed of cattle. Unfortunately, however, similar studies in Sahiwal cow (the principle native dairy breed of Pakistan) as yet are almost non-existent.

In Pakistan, a thorough investigation of a preceding study revealed that the prevalence of mastitis (clinical and sub-clinical) triggered by pathogenic microorganisms in cattle and buffaloes was 46.72% (Athar 2007; Beheshti *et al*. 2010). Different indirect screening tests for diagnosis include SSC count through the authentic counter, California mastitis test, ELISA test, and Surf-field mastitis test (Batavani *et al*. 2007; Muhammad *et al*. 2010) which reflects a greater degree of discrepancies in results. Such a scenario helps this malaise continue to increase. Authentic indicators are necessary to implement whereas tumor necrosis factor-alpha (*TNF-α*) identified direct relation with mastitis.

The pathogenic microorganisms can trigger the immune response in the mammary tissue (Oviedo *et al*. 2007; Wellnitz and Bruckmaier 2012). The Toll-like receptors are considered as first-line of defense because it recognizes pathogenic microorganism and leads to the activation of transcription factors and triggering the expression of pro-inflammatory molecules (Pasare and Medzhitov 2004). In the early immune response, mammary epithelial cells are responsible for the activation of cytokines i.e., interleukins, tumor necrosis factor-alpha (*TNF*-α), and interferon-gamma (*IFN*-γ) and production of other factors having antimicrobial activities. Among these cytokines, *TNF*-α is a fundamental mediator in the inflammatory response. It stimulates vasodilation and an increase in vascular permeability, promoting the recruitment of leukocytes and serum proteins to the infection site (Medzhitov 2007; Brenaut *et al*. 2014). For this and other reasons, *TNF*-α is considered a key component of the innate immune system.

TNF-α is a pleiotropic cytokine associated with systemic inflammation and is mainly secreted by activated macrophages and monocytes. The precursor molecule of *TNF*-α is 26 kDa which undergoes further processing to synthesize a 17 kDa carboxy-terminal protein by cleavage of the bond between Ala^{76}-Val^{77} and secreted to function in a paracrine manner (Bannerman 2009; Moyes *et al*. 2009; Sennikov *et al*. 2014). Resistance to bovine mastitis is a multifactorial trait and immunity genes are key indicators towards an understanding of disease cascade. Keeping in view the potential linkage of *TNF-α* with disease condition, the current study was planned to characterize the 5'upstream region and assess the relative mRNA expression of *TNF-α* gene in clinical and subclinical mastitis in Sahiwal cows.

Materials and Methods

Experimental animals

The current study was conducted on Sahiwal cows suffering from clinical and subclinical mastitis. In this study, 40 Sahiwal cows (n=40) were selected from different Government and private dairy farms of Punjab, Pakistan and divided into three groups i.e., Sahiwal clinical mastitis (SCM: n=15) group, Sahiwal subclinical mastitis (SSM: n=15) group, and Sahiwal Non-mastitic (SNM: n=10) group. For the diagnosis of the subclinical mastitis, Surf-field mastitis test (Muhammad *et al*. 2010) was performed as a point-of-case test.

Blood collection

The blood (2–3 mL) was drawn from the jugular vein of selected animals and transferred to blood collection vials containing EDTA (anti-coagulant) and was mixed gently for proper mixing to avoid coagulation. Then the vials were immediately placed on ice and transferred to the laboratory.

DNA extraction and quantification

DNA was extracted from blood samples by the phenol-chloroform isolation method (Sambrook and Russell 2001). DNA quantification was done with the help of Nanodrop (Thermo Scientific Spectrophotometer ND-2000). 1 µL of the sample was utilized to determine the concentration of DNA by Nanodrop. All DNA samples were adjusted at the same concentration (50 ng/ µL) for PCR.

Primer designing and amplification

The region was determined for the amplification of *TNF-α* gene: TNF1 that encompasses partial 5´ upstream region. The primers were designed by using sequence retrieved from NCBI **(**XM_005223596) with the help of online software Primer3 (http://wwwgenome.wi.mit.edu/cgi-bin/primer/primer3 www.cgi). The details of primer sequence are given in Table 1. A total of 25 µL PCR reaction solution was prepared containing template DNA (50 ng/µL), Primers (10 pmol), $MgCl_2$ (2.0 mmol), 1X Buffer, dNTPs (0.25 mmol), and TaqDNA polymerase (0.5 U). The amplification was carried out by heating mixture at 94ºC for 5 min (initial denaturation), followed by 35 cycles of final denaturation at 94ºC for 30 sec, annealing at 56ºC for 30 sec, extension at 72ºC for 30 sec with final extension for 10 min. The amplicons were separated on 1.2% agarose gel. Then, amplicons were subjected to commercial sequencing using dye-labeled dideoxy terminator cycle sequencing using ABI prism 3130 XL Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA, USA).

RNA isolation and cDNA synthesis

RNA was isolated from fresh blood samples through the RNA purification kit (Thermo Scientific, USA), according to the manufacture's protocol. The RNA concentration was checked through Nanodrop spectrophotometer by

measuring absorbance at 260/280 nm. The cDNA was synthesized using the Revert Aid First Stand cDNA synthesis kit (Thermo Scientific, Pittsburg, PA, USA) as per the instructions of manufacturer.

Real-time qPCR analysis

Real-time qPCR was performed in 96-well plates (Rotor-Gene® Q). The Gene-specific Taqman primer-probe PCR master mix kit (BioRad, Hercules, CA, USA) was used for the amplification. The primer used for the assessment of the expression of the target gene (*TNF-α*) and of the endogenous control (*GAPDH*) have been described in the literature (Table 1). The sequence of primers and corresponding gene showed 100% similarity, so it was not necessary to redesign the primers. The real-time qPCR assays were performed in triplicate for each sample of the target gene and housekeeping gene that is Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) used as an endogenous control. The reaction mixture containing 2X Taqman master mixtures 12.5 µL (Thermo Scientific, Pittsburg, PA, USA), 2 µL Taqman primer-probes and 2 µL template (cDNA) was prepared. The thermal profile used for this was as follows: 95ºC for 10 min, then 35 cycles of 94ºC for 15 sec, 60°C for 15 sec and 72ºC for 15 sec followed by denaturation at 72ºC for 10 min. The cycle threshold (Ct) values were obtained and expressed as fold change calculated by Livak method (Livak and Schmittgen 2001). The ΔCt value was obtained by subtracting the mean Ct value of target gene (*TNF-α*) from the Ct value of endogenous *GAPDH* gene (reference gene). ΔΔCt value was calculated by subtracting the ΔCt value of target from the Calibrator and then fold change was calculated by using formula $2^{-\Delta\Delta Ct}$.

Statistical analysis

The data obtained in the study were analyzed statistically using SPSS (v6.1). Correlation among the variables was also performed through R-studio (R v3.6.2), while response surface methodology was carried out via Box Behnken design with the help of design expert software (v12, USA).

Results

The current study was designed to identify single nucleotide polymorphism (SNPs) in 5´ upstream region of *TNF-α* gene of Sahiwal cows and their association with differential expression profiling toward mastitis susceptibility. DNA was extracted from blood samples and then 484 bp fragment of the *TNF-α* gene was amplified by PCR (Fig. 1A; Fig. 1B). Polymorphism analysis revealed that there is a change in nucleotide at position 130 (A>G) in both clinical and subclinical mastitic samples but not in non-mastitic cow samples (Fig. 1C). The DNA sequence of the gene is available in GenBank with Accession number MT_919286.

Table 1: Details of primers used for the amplification (TNF1, TNF2) and TaqMan primer-probes

Primer Name	Primer sequence/ TaqMan assay ID	Species	Amplicon length (bp)
TNF1-F	5´ CAGCACAGCTTCCTCTGAGTT 3´	Bovine	484
TNF1-R	5´ CGCTCTGGGAGCTTCTGTT 3´	Bovine	
TNF-α	Bt03259156 (20x, 250)	Bovine	69
GAPDH	Bt03210913 (20x, 250)	Bovine	66

Table 2: Design approach for determining the optimization of mastitis disease in Sahiwal cow via Box–Behnken Design

Parameters	Coded Symbol	Range		
		-1	0	1
Fold Change	A	1.8	9.53	35
TNF-α	B	20.25	22.36	24.22
GAPDH	C	25.25	25.42	25.61

The relative mRNA expression of the *TNF-α* gene in clinical mastitis, subclinical mastitis, and non-mastitic Sahiwal cows was carried out through real-time qPCR. In clinical and subclinical mastitis, mRNA expression was observed with the highest fold change in the clinical (56.8 times) and 12.55 times in subclinical, but substantial variation was also noted in *TNF-α* expression within the groups. A remarkable decrease in *TNF-α* mRNA expression was also noted in healthy animals with the highest fold change being 2.3 (Fig. 2). The findings of this study showed that *TNF-α* gene expression has been found to be significantly up-regulated in both clinical and subclinical mastitis as compared to that in non-mastitic cows.

Optimization of fold Change of TNF-α and GAPDH against Sahiwal cow clinical Mastitis, Sahiwal cow sub-Clinical Mastitis and non-mastitic Sahiwal cow via response surface methodology

For all the parameters which were determined in the study, the effect of fold change, TNF-α and GAPDH was assessed based on response surface methodology via Box Behnken design (Table 2). The data were applied on the following equation:

$$Y = \beta_o + \sum_{i=1}^{3} \beta_{ii} F_i + \sum_{i=1}^{3} \beta_{ii} F_i^2 + \sum \sum_{i<j=1}^{3} \beta_{ij} F_i F_j$$

where "*Y"* was the response variable, "β_0" was the intercept constant, "*βi"*, "*βii"*, "*βij"* were the regression coefficients of "F_1", "F_2", "F_3", "F_i", "F_j" were coded values of independent variables.

Based upon this design, the analysis of variance was performed which described the effect of all the selected parameters against clinical mastitis (Table 3; Fig. 3), subclinical mastitis (Table 4 and Fig. 4) and Sahiwal non-mastitic cow (Table 5, 6 and Fig. 5). The regression equation clarifies the effect of all parameters applied on the three different treatments of Sahiwal cows.

Discussion

Bovine mastitis is primarily an inflammatory response of

Table 3: Analysis of variance of optimization parameters against Sahiwal cow clinical mastitis (SCM) via Response Surface Methodology

Source	Sum of Squares	df	Mean Square	F-value	P-value	
Model	218.58	9	24.29	6.62	0.0104	Significant
A-Fold Change	29.57	1	29.57	8.06	0.0250	
B-TNF-α	52.69	1	52.69	14.37	0.0068	
C-GAPDH	1.49	1	1.49	0.4058	0.0444	
AB	4.49	1	4.49	1.23	0.3048	
AC	20.88	1	20.88	5.70	0.0484	
BC	52.35	1	52.35	14.28	0.0069	
A^2	33.06	1	33.06	9.02	0.0199	
B^2	23.96	1	23.96	6.53	0.0378	
C^2	2.73	1	2.73	0.7438	0.0170	
Residual	25.66	7	3.67			
Lack of Fit	6.85	3	2.28	0.4857	0.7103	Not significant
Pure Error	18.81	4	4.70			
Cor Total	244.24	16				

R^2 = 89.49%

$SCM = 8.17 + 1.80A + 1.76B + 0.8644C + 0.9331AB – 2.28AC – 3.18BC – 2.80A^2 + 1.85B^2 – 0.8048C^2$

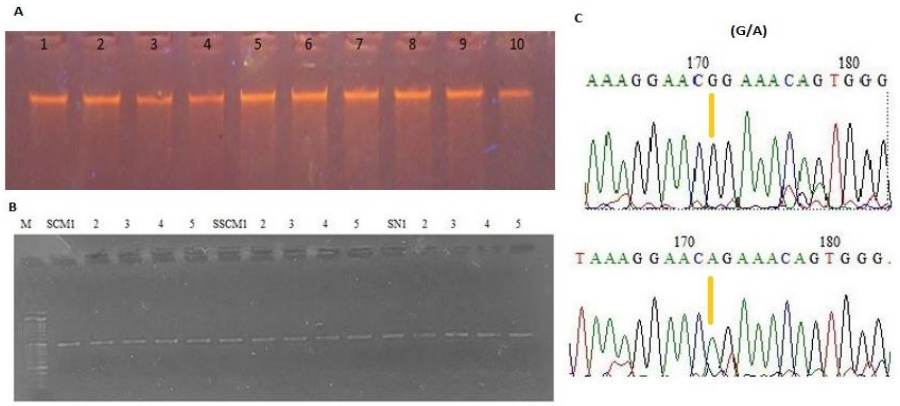

Fig. 1: A. Gel electrophoresis results of extracted DNA, **B**. PCR amplification of *TNF-α* gene fragment of clinical (SCM1-5), subclinical (SSCM1-5) and non-mastitic Sahiwal cows (SNM1-5)**, Lane M:** Marker 50bp (Fermentas), **C**. Electropherogram of position 130 of *TNF-α* showing substitution (G) in mastitis sample instead of (A) in samples of non-mastitic cows

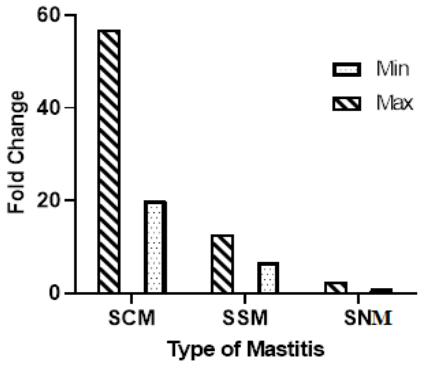

Fig. 2: Minimum and maximum relative mRNA expression of *TNF-α* in Clinical, subclinical and normal Sahiwal cow samples
SCM= Sahiwal clinical mastitis
SSM= Sahiwal subclinical mastitis
SNM= Sahiwal non-mastitic

mammary gland tissue against pathogenic microorganisms (Barkema *et al*. 1998; Fox 2009; Mpatswenumugabo *et al*. 2017). The inflammatory response is regulated by a network of cytokines during udder infection. It has been revealed that intramammary (IM) reaction stimulates a differential innate immune response (Riollet *et al*. 2001; Bannerman *et al*. 2004; Bharathan and Mullarky 2011). It has been reported that *TNF-α* is present in mastitic animal milk infected with gram-negative bacteria instead of other cytokines like *IFN-γ*, *IL-1* and *IL-8* and used as potential genetic marker for the diagnosis of mastitis in dairy animals (Bannerman *et al*. 2004). *TNF-α* is a pro-inflammatory cytokine that triggers the process of inflammation and plays a significant role in the host defense mechanism against udder infection (Persson *et al*. 2011; Hayashi *et al*. 2013).

The 5´ upstream region of *TNF-α* has been very well characterized both in humans and cattle (Yea *et al*. 2001; Bojarojć-Nosowicz *et al*. 2011), but the polymorphism and its association with disease incidence have not been reported in Sahiwal cattle so far. In this investigation, an attempt has been made to explore the single nucleotide polymorphism (130, A>G) in 5´ upstream region and its association with mastitis susceptibility in Sahiwal cattle. Earlier, different studies revealed a significant association of genetic variation in the *TNF* promoter region with disease resistance, susceptibility, and progression (Deshpande *et al*. 2005; Konnai *et al*. 2006; Kumar *et al*. 2019).

In the present study, polymorphism in *TNF-α* gene had

Table 4: Analysis of variance of optimization parameters against Sahiwal cow subClinical mastitis (SSM) via Response Surface Methodology

Source	Sum of Squares	df	Mean Square	F-value	P-value	
Model	286.99	9	31.89	5.33	0.0191	Significant
A-Fold Change	41.86	1	41.86	7.00	0.0332	
B-TNF-α	57.19	1	57.19	9.56	0.0175	
C-GAPDH	0.4278	1	0.4278	0.0715	0.7968	
AB	18.32	1	18.32	3.06	0.1236	
AC	20.34	1	20.34	3.40	0.1077	
BC	59.99	1	59.99	10.03	0.0158	
A^2	64.61	1	64.61	10.80	0.0134	
B^2	27.92	1	27.92	4.67	0.0675	
C^2	0.3535	1	0.3535	0.0591	0.8149	
Residual	41.87	7	5.98			
Lack of Fit	16.46	3	5.49	0.8640	0.5290	Not significant
Pure Error	25.40	4	6.35			
	328.86	16				

R^2 = 87.27%

SSM = 8.33 + 2.03A + 1.81B + 0.6949C + 1.88AB – 2.25AC – 3.41BC – $3.92A^2$ + $2.00B^2$ – $0.2898C^2$

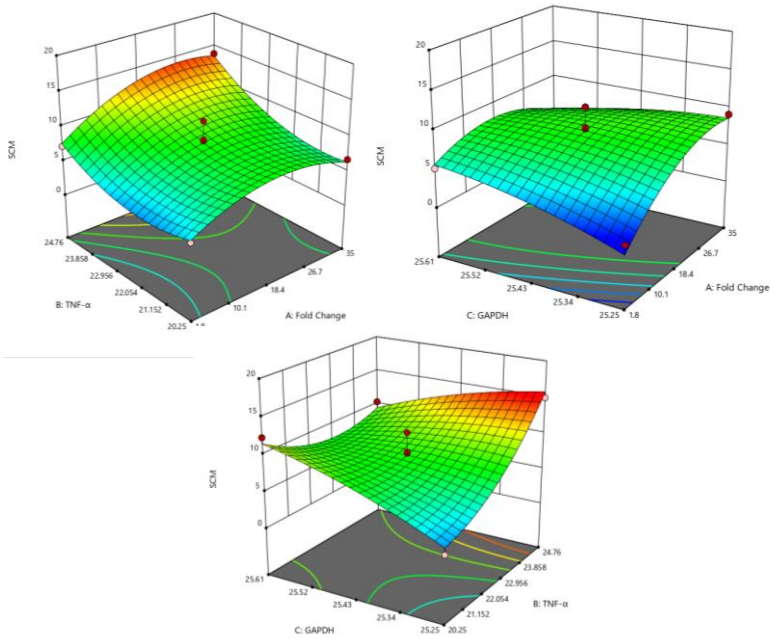

Fig. 3: Optimization of parameters against SCM
SCM= Sahiwal clinical mastitis

a complex influence on relative mRNA expression in cows infected with mastitis. Messenger RNA expression of the *TNF-α* gene was significantly higher in Sahiwal cow with clinical and subclinical mastitis as compared to non-mastitic Sahiwal cows, reflecting an association of this gene with innate immunity efficiency caused by mastitis. The results suggest that the change in a unit of ΔCt is responsible for higher fold change and consequently a higher inflammatory response. Previously, Burvenich *et al.* (2003) have described that the variation in the gene expression profile may be attributed to a score of factors: (a) the intensity of infection, (b) the magnitude of an inflammatory response in individual animal, (c) detection by the highly sensitive real-time qPCR which reveals even the slightest variation between samples. The surface plots developed based upon this analysis also depicts that the determined parameters had a significant impact on mastitis disease incidence in clinically mastitic Sahiwal cows. A similar trend was observed in SSM and SNM where the coefficient of determination was 87% and 85%, respectively. Therefore, these factors also showed a significant trend towards mastitis incidence in Sahiwal cows.

Our results are in synergy with the findings of previous studies that utilized real-time qPCR to examine the gene expression of numerous cytokines in response to *E. coli* and *S. aureus* in Holstein cows. The results elucidate that the target cytokine gene (*TNF-α*) expression is higher in mastitic cows as compared to the non-mastitic Sahiwal cows (Riollet *et al.* 2001; Lee *et al.* 2006). Some other experiments done in Crossbred cows (Holstein Friesian=Jersey with Hariana=

Table 5: Analysis of variance of optimization parameters against Sahiwal non-mastitic (SNM) cow via Response Surface Methodology

Source	Sum of Squares	df	Mean Square	F-value	P-value	
Model	225.66	9	25.07	4.69	0.0269	Significant
A-Fold Change	34.82	1	34.82	6.52	0.0379	
B-TNF-α	44.27	1	44.27	8.29	0.0237	
C-GAPDH	0.0001	1	0.0001	0.0000	0.9965	
AB	35.82	1	35.82	6.70	0.0360	
AC	5.66	1	5.66	1.06	0.3374	
BC	40.64	1	40.64	7.61	0.0282	
A^2	45.11	1	45.11	8.44	0.0228	
B^2	22.46	1	22.46	4.20	0.0795	
C^2	0.1038	1	0.1038	0.0194	0.8931	
Residual	37.40	7	5.34			
Lack of Fit	17.13	3	5.71	1.13	0.4382	Not significant
Pure Error	20.28	4	5.07			
Cor Total	263.06	16				

R^2 = 85.78%

$SN = 6.51 + 1.73A + 1.58B + 0.3779C + 2.63AB – 1.19AC – 2.81BC – 3.27A^2 + 1.79B^2 + 0.1570C^2$

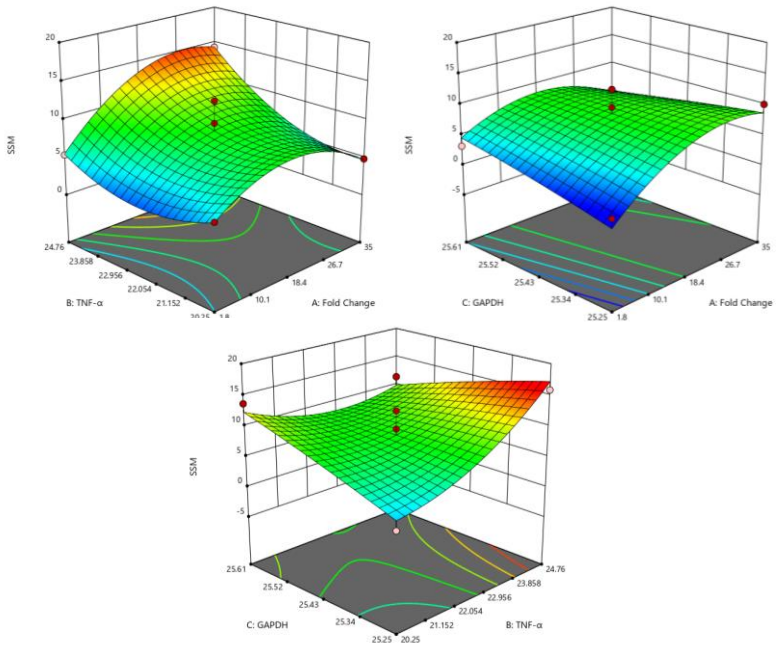

Fig. 4: Optimization of parameters against SSM
SSM= Sahiwal subclinical mastitis

Brown Swiss) showed up-regulation of *TNF-α* gene expression after the subsequent induction of mastitis by LPS (Lipo-polysaccharide) exposure which is the key virulence factor of Gram-negative bacteria (Blum *et al.* 2000; Kahl *et al.* 2009; Ranjan *et al.* 2015).

The findings revealed that the immune response of mastitis affected groups (clinical and subclinical) consisting a higher *TNF-α* mRNA expression along with up-regulation of *IL-8*, *IL-6*, *IL-12* & interferon (mentioned in other studies) serves as a crucial defense mechanism, which is lacking in non-mastitic Sahiwal cows (Ranjan *et al.* 2015). The mRNA expression of *TLR-2 TNF-α, IL-1β,* and *IL-8* was respectively 13.34, 7.15, 62.49 and 26 times higher in subclinically mastitic buffaloes, as were also observed in cattle (Fonseca *et al.* 2015; Tanamati *et al.* 2019). Our findings seem to support the research of other authors which suggests that immune system genes are important to characterize the action mechanism of the immune system that occurs in clinical and subclinical mastitis.

Conclusion

The results of present study indicate that polymorphism in promoter region of *TNF-α* at position130 (A>G) might be associated with mastitis susceptibility and influences relative mRNA expression of this gene in mastitis affected Sahiwal cows. The fold change of *TNF-α* was 35, 9.35, and 1.8 times in clinical mastitis, subclinical mastitis, and non-mastitic milk samples, respectively. Such higher expressions favor use of *TNF-α* gene as an accurate marker of severity

Table 6: Predicted and experimental values of optimization parameters via Box-Behnken Design

Runs	Fold Change	TNF-α	GAPDH	SCM		SSM		SNM	
				Observed values	Predicted values	Observed values	Predicted values	Observed values	Predicted values
1	1.8	22.505	25.25	1.4	1.21	1.1	0.95	1.5	1.01
2	18.4	20.25	25.25	2.5	2.22	2.6	2.21	2.2	2.07
3	35	22.505	25.61	3.7	3.12	3.2	3.11	3.4	3.32
4	1.8	20.25	25.43	4.3	4.28	4.5	4.59	4.7	4.52
5	18.4	22.505	25.43	5.6	5.43	6.7	6.56	5.4	5.32
6	18.4	22.505	25.43	7.5	7.45	7.8	7.48	6.23	6.11
7	18.4	22.505	25.43	8.7	8.65	9.6	9.39	7.71	7.47
8	35	22.505	25.25	9.12	9.09	10.11	10.03	8.65	8.23
9	18.4	24.76	25.61	10.31	10.11	11.45	11.34	9.87	9.19
10	18.4	22.505	25.43	11.54	11.42	12.53	12.11	10.12	10.01
11	18.4	20.25	25.61	12.31	12.21	13.67	13.41	11.43	11.12
12	35	24.76	25.43	13.87	13.76	14.32	14.56	12.87	12.76
13	18.4	24.76	25.25	14.97	14.95	15.87	15.43	13.39	13.78
14	18.4	22.505	25.43	8.87	8.81	6.43	6.13	4.32	3.97
15	1.8	24.76	25.43	7.21	7.17	5.39	5.11	3.31	3.10
16	35	20.25	25.43	6.72	6.45	4.87	4.23	2.29	2.18
17	1.8	22.505	25.61	5.12	5.01	3.21	3.03	1.01	0.78

Based on analysis of variance applied on this model, it became obvious that this model has shown significant response at 5% level of significance while this model is also very suitable and reproducible due to having very less lack of fit (P>0.05). The co-efficient of determination (R^2) also confirms that with 89% surety the data regarding SCM is highly significant and has potential application under various conditions. Thus, the optimum parameters have also been defined as shown in Table 3 and Fig. 3

SCM= Sahiwal clinical mastitis

SSM= Sahiwal subclinical mastitis

SNM=Sahiwal non-mastitic

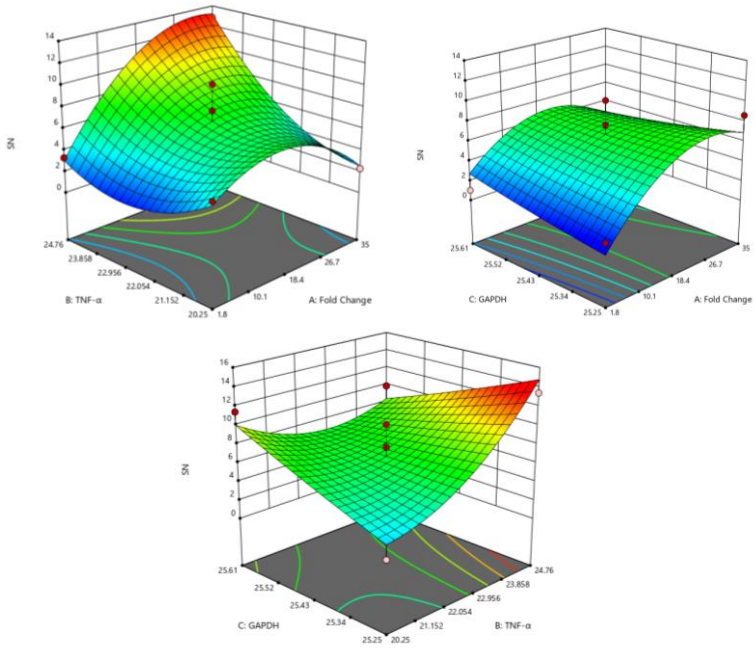

Fig. 5: Optimization of parameters against SNM

SNM= Sahiwal non-mastitic

of mastitis which is time and cost saving authentic approach to be used as diagnostic and research purposes. The findings of the present study would help scientific community to understand the genetic mechanisms underlying *TNF-α* mediated mastitis susceptibility.

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All the authors contributed equally.

References

Athar M (2007). Preparation and evaluation of inactivated polyvalent vaccines for the control of mastitis in dairy buffaloes. *Ph.D. Thesis*, Department of Clinical Medicine and Surgery, Faculty Veterinary Science, University Agriculture Faisalabad, Pakistan

Bannerman D (2009). Pathogen-dependent induction of cytokines and other soluble inflammatory mediators during intramammary infection of dairy cows. *J Anim Sci* 87:10–25

Bannerman DD, MJ Paape, JW Lee, X Zhao, JC Hope, P Rainard (2004). *Escherichia coli* and *Staphylococcus aureus* elicit differential innate immune responses following intramammary infection. *Clin Diagn Lab Immunol* 11:463–472

Barkema HW, YH Schukken, TJGM Lam, ML Beoboer, G Benedictus (1998). Management practices associated with low, medium and high somatic cell counts in bulk milk. *J Dairy Sci* 81:1917–1927

Batavani RA, S Asri, H Naebzadeh (2007). The effect of subclinical mastitis on milk composition in dairy cows. *Iran J Vet Res* 8:205–211

Beheshti R, J Shaieghi, B Eshratkhah, JG Ghalehkandi, SN Maheri (2010). Prevalence and etiology of subclinical mastitis in buffalo of the Tabriz region, Iran. *Global Vet* 4:299–302

Bharathan M, IK Mullarky (2011). Targeting mucosal immunity in the battle to develop a mastitis vaccine. *J Mammary Gland Biol Neoplasia* 16:1409–1910

Blum J, H Dosogne, D Hoeben, F Vangroenweghe, H Hammon, R Bruckmaier, C Burvenich (2000). Tumor necrosis factor-α and nitrite/nitrate responses during acute mastitis induced by *Escherichia coli* infection and endotoxin in dairy cows. *Domest Anim Endocrin* 19:223–235

Bojarojć-Nosowicz B, E Kaczmarczyk, A Stachura, M Kotkiewicz (2011). Polymorphism in the promoter region of the tumor necrosis factor-alpha gene in cattle herds naturally infected and uninfected with the bovine leukemia virus. *Pol J Vet Sci* 14:671–673

Brenaut P, L Lefevre, A Rau, D Laloe, G Pisoni, P Moroni (2014). Contribution of mammary epithelial cells to the immune response during early stages of a bacterial infection to *Staphylococcus aureus*. *Vet Res* 45:10–16

Burvenich C, V Merris, J Mehrzad, AD Fraile, L Duchateau (2003). Severity of *E. coli* mastitis is mainly determined by cow factors. *Vet Res* 34:521–564

Deshpande A, JP Nolan, PS White, YE Valdez, WC Hunt, CL Peyton (2005). TNF-α promoter polymorphisms and susceptibility to human papillomavirus 16–associated cervical cancer. *J Infect Dis* 191:969–976

Cobirka M, T Vladimir, S Petr (2020). Epidemiology and classification of mastitis. *Animals*10; Article 2212

Dua K (2001). Incidence, etiology and estimated economic losses due to mastitis in Punjab and in India-An update. Indian. *Dairyman* 53:41–48

Ebrahimi A, KHP Kheirabadi, F Nikookhah (2007). Antimicrobial susceptibility of environmental bovine mastitis pathogens in west central Iran. *Pak J Biol Sci* 10:3014–3016

Fonseca I, FF Cardoso, RH Higa, PF Giachetto, HM Brandao, MAVP Brito, MBD Ferreira, SEF Guimaraes, MF Martins (2015). Gene expression profile in Zebu dairy cows (*Bos taurus indicus*) with mastitis caused by *Streptococcus agalactiae*. *Livest Sci* 180:47–57

Fox LK (2009). Prevalence, incidence and risk factors of heifer mastitis. *Vet Microbiol* 134:82–88

Hayashi K, V Piras, S Tabata, K Katsuda, E Zhang, Y Kiku, K Sugawar, T Ozawa, T Matsubara, T Ando, T Obayashi, T Ito, T Yabusaki, K Kudo, H Yamaoto, M Koiwa, T Oshida, Y Tagawa, K Kawai (2013). A systems biology approach to suppress TNF-induced proinflammatory gene expressions. *Cell Commun Signal* 11:84

Kahl S, TH Elsasser, M Proszkowiec-Weglarz, EE Connor (2009). Association of tumor necrosis factor-alpha (TNF-α) gene promoter polymorphisms with hyper-responiveness to endotoxin (LPS) I calves. *Joint Abst Amer Dairy Sci Soc Anim Sci* 87:13

Konnai S, T Usui, M Ikeda, J Kohara, TI Hirata, K Okada (2006). Tumor necrosis factor-alpha genetic polymorphism may contribute to progression of bovine leukemia virus-infection. *Microb Infect* 8:2163–2171

Kumar A, SK Mishra, S Lavakumar, VS Karan, K Namita, S Monika, M Mukesh, SK Niranjan, K Avnish, RS Kataria (2019). Detection of polymorphism in the promoter region of TNF-alpha gene of water buffalo (*Bubalus bubalis*) and its association with disease resistance. *Ind J Anim Res* 53:1572–1576

Lee JW, DD Bannerman, MJ Paape, MK Huang, X Zhao (2006). Characterization of cytokine expression in milk somatic cells during intramammary infections with *Eschericha coli* or *Staphylococcus aureus* by real-time PCR. *Vet Res* 37:219–229

Livak KJ, TD Schmittgen (2001). Analysis of relative gene expression data using Real Time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 25:402–408

Medzhitov R (2007). Recognition of microorganisms and activation of the immune response. *Nature* 449:819–826

Moyes KM, JK Drackley, JL Salak-Johnson, DE Morin, JC Hope, JJ Loor (2009). Dietary-induced negative energy balance has minimal effects on innate immunity during a *Streptococcus uberis* mastitis challenge in dairy cows during mid-lactation. *J Dairy Sci* 92:4301–4316

Mpatswenumugabo JP, LC Bebora, GC Gitao, VA Mobegi, B Iraguha, O Kamana, B Shumbusho (2017). Prevalence of subclinical mastitis and distribution of pathogens in dairy farms of Rubavu and Nyabihu Districts, Rwanda. *J Vet Med* 2017; Article 8456713

Muhammad G, A Naureen, MN Asi, M Saqib (2010). Evaluation of a 3% surf solution (surf field mastitis test) for the diagnosis of subclinical bovine and bubaline mastitis. *Trop Anim Health Product* 42:457–464

Oviedo BJ, JJ Valdez-Alarcon, M Cajero-Juarez, A Ochoa-Zarzosa, JE Lopez-Meza, A Bravo-Patino, VM Baizabal-Aguirre (2007). Innate immune response of bovine mammary gland to pathogenic bacteria responsible for mastitis. *J Infect* 54:4091–4399

Pasare C, R Medzhitov (2004). Toll-like receptors: Linking innate and adaptive immunity. *Microb Infect* 6:1382–1387

Persson Y, J Nyman, UG Andersson (2011). Etiology and antimicrobial susceptibility of udder pathogens from cases of subclinical mastitis in dairy cows in Sweden. *Acta Vet Scand* 53:36

Ranjan S, B Bhushan, M Panigrahi, A Kumar, R Deb, P Kumar, D Sharma (2015). Association and expression analysis of single nucleotide polymorphisms of partial tumor necrosis factor alpha gene with mastitis in crossbred cattle. *Anim Biotechnol* 26:98–104

Rehman A, JD Luan, AC Abbas, H Imran (2017). Livestock production and population census in Pakistan: Determining their relationship with agricultural GDP using econometric analysis. *Inf Proc Agric* 4:168–177

Riollet C, P Rainard, B Poutrel (2001). Cell subpopulations and cytokine expression in cow milk in response to chronic *Staphylococcus aureus* infection. *J Dairy Sci* 84:1077–1084

Sambrook J, DW Russell (2001). *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York, USA

Sender S, KK Agnieszka, P Adrianna, GAH Karima, O Jolanta (2013). Genetic basis of mastitis resistance in dairy cattle-A review. *Ann Anim Sci*. 13:663–673

Sennikov SV, FF Vasilyev, JA Lopatnikova, NS Shkaruba, AN Silkov (2014). Polymorphism in the tumor necrosis factor receptor gene affect the expression levels of membrane bound type I and type II receptors. Mediat Inflamm 2014; Article 745909

Sharma N, SK Maiti (2010). Incidence, etiology and antibiogram of sub clinical mastitis in cows in durg, Chhattisgarh. *Ind J Vet Res* 19:45–54

Tanamati F, NB Stafuzza, DFJ Gimenez, AAS Stella, DJA Santos, MIT Ferro, Albuquerque, E Gasparino, H Tonhati (2019). Differential expression of immune response genes associated with subclinical mastitis in dairy buffaloes. *Animal* 13:1651–1657

Wellnitz O, RM Bruckmaier (2012). The innate immune response of the bovine mammary gland to bacterial infection. *Vet J* 192:148–210

Yea SS, YI Yang, WH Jang, YJ Lee, HS Bae, KH Paik (2001). Association between TNF-α promoter polymorphism and *Helicobacter pylori* cag A subtype infection. *J Clin Pathol* 54:703–706