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SHORT COMMUNICATION

Association of Bovine Tumor Necrosis Factor Alpha Gene Polymorphism with Mastitis in Nili Ravi Buffaloes

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ABSTRACT

The current research was designed to find out polymorphic changes in complete gene sequence of tumor necrosis factor alpha (TNF-α), which is involved in inducing mastitis in Nili Ravi buffaloes. Total 30 blood samples were collected having clinical (n=10), subclinical (n=10) mastitis and normal (n=10) animals. DNA was extracted, TNF-α gene was amplified and sequenced. Homology analysis of TNF-α gene sequences using Finch TV tool revealed total of eleven SNP's in subclinical mastitic buffaloes and twelve in clinical mastitic buffaloes, including insertion, heterozygosity, transitional and some silent mutations; in intronic region. In subclinical mastitis samples, insertion of "G" at the position 279, transition mutation of (A/G) at position 1022, Heterozygosity of (A/G) at 1023 cite, transition mutation of (A/G) at position 1129, deletion of "C" at position 1741 and transition of (A/G) were found at cite 2091 of this gene, respectively. In the clinical mastitis samples, there was an insertion of "G" at the position 279, transition mutation of (A/G) at position 1022 and Heterozygosity of (A/G) at 1023 cite were found. All these SNPs were not found in sequences of normal animals. The current genome association study showed the potential correlation between these significant polymorphisms and incidence of clinical and subclinical mastitis in Nili Ravi buffaloes.

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INTRODUCTION

Mastitis is one of the multi-etiological odious ailments of the milching animals, influencing the quality and quantity of milk. It is a mammary glands inflammation, which can be infectious or non-infectious (Firyal *et al*., 2017). According to the field surveys mastitis is the most prevalent disease causing high economical loss by influencing milk production rate in Pakistan. It is not only the cause of the reduction in milk yield but also cause undesirable compositional changes which affects the manufacturing procedure of many dairy products (Metzner *et al*., 2014).

Mastitis is caused by the complex interaction of hosts (cow, buffalo etc.), agents (microorganisms) and environment. It is reported that many microorganisms such as yeast, fungi and bacteria have association with mastitis but bacteria are the most frequently isolated infectious agent from cases of bovine and bubaline mastitis. Prevalence of clinical mastitis in Nili Ravi buffalo is 23.78% which is greater than cattle as in cattle it prevalence is 15.38% (Khan *et al*., 2015).

It is very complicated to comprehend this disease because with numerous environmental factors, andgenetic factors are involved in its etiology (Carvajal *et al*., 2013; Firyal *et al*., 2018). The resistance and the susceptibility to mastitis is a complicated trait prejudice by the genetic variation of animals. The main key factor in the immune mechanism of the mammary gland is the polymorphism in immunity genes (Ibeagha-Awemu *et al*., 2008).

Innate and acquired immunity are the two key defense mechanisms used for the protection of mammary glands (Mesquita *et al*., 2012). Tumor Necrosis Factor alpha is one of the main cells signaling adipokine that is involved in the systematic inflammatory immune response. TNF-α is a pyrogen, so it results in the

stimulation of fever while inducing proliferation, differentiation and activation of immunity cells i.e. B lymphocytes, NK (natural killer). Many other cytokines are also released upon its onset (Wojdak-Maksymiec *et al*., 2013) enhancing the chemotactic and phagocytic response of immune system. TNF- α of Nili Ravi buffalo is a group member of cytokines, which trigger the specific immune system. Gene encoding TNF-α contains 4 exons and 5 introns.

The present study for polymorphism analysis of TNF-α was designed to determine the genetic polymorphism in complete TNF-α gene of mastitic buffalo (Nili Ravi) and its association to resistance and susceptibility towards mastitis. These genetic variations allow the animal breeders to evaluate the genetic predisposition of Nili Ravi buffaloes to develop the mastitis.

MATERIALS AND METHODS

Blood samples from 30 Nili Ravi buffalos were taken, having clinical (n=10), subclinical (n=10) mastitis & normal (n=10) animals. Surf field mastitis test was carried out for the identification of subclinical mastitic and normal buffaloes, whereas clinically mastitic buffaloes were distinguished by considering clinical mastitic symptoms. For the detection of subclinical mastitis, Surf Field Mastitis Test was performed at animal site (Muhammad *et al*., 1995). DNA extraction from blood was carried out using organic extraction method, followed by DNA quantification (i.e. gel electrophoresis and nanodrop).

Total 5 primers (Table 1) were designed by using Primer 3 bioinformatics tool. All these primers were optimized using different protocols and a set recipe was obtained for each primer. The amplification of DNA samples was done one by one using all these five primers through optimized protocol. The amplicons were subjected to agarose gel electrophoresis by using 100 kb ladder and then amplicons were sent for the sequencing using BigDye terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, CA, USA). The sequencing analysis was carried out by using Bioinformatics software Finch TV (version 1.40).

RESULTS AND DISCUSSION

SNPs identified in TNF-α gene sequence of subclinical and clinical mastitis samples are presented in Tables 1 and 2. Comparison of subclinical mastitis samples with the normal NCBI sequence samples showed polymorphisms at different site. Total of 6 polymorphic sites were found in subclinical and clinical mastitis samples, while 5 were same in all the samples, whereas 6th polymorphism was found only in clinical samples.

In subclinical mastitis samples, insertion of "G" at the position 279, transition mutation of (A/G) at position 1022, Heterozygosity of (A/G) at 1023 cite, transition mutation of (A/G) at position 1129, deletion of "C" was found at position 1741, transition of (A/G) was found at cite 2091of this gene, respectively.

Comparison of clinical mastitis samples with the normal sequence taken directly from NCBI also showed mutations at many cites i.e. there was an insertion of "G" at the position 279, transition mutation of (A/G) at position 1022, Heterozygosity of (A/G) at 1023 cite, At position 1129 there was transition mutation of "A" while reference sequence showing "G" at this position. The deletion of "C" was found at position no. 1741.

Table 1: Primers used in study

Sr. No	Primer name	5'- 3' Sequence	Tm (°C)	Product size (bp)
1.	BT1-F	ATAAAGCCCCTCCCATTTCTAA	54	196
	BT1-R	GGATTCTGAGTGGGCTTCTCTA	54	
2.	BT2-F	GGTCCCGTATTCACAGAGGTAA	54	485
	BT2-R	GTGCTCATGGTGTCTTTTTCAG	54	
3.	BT3-F	AGACACCATGAGCACCAAAAG	54	597
	BT3-R	ATGCCAGACACACTTAGCTTCA	54	
4.	BT4-F	CATGTGGAAGGAACTCAATGAA	56	558
	BT4-R	TACTGTCTCTGTCTGCCCTCAG	56	
5.	BT5-F	CTGAGGGCAGACAGAGACAGTA	56	697
	BT5-R	AAGGTAACTGAGGTGGGAGAGG	56	

Fig. 1: PCR Amplification of TNF-α gene of Nili Ravi buffalo clinical mastitis using BT1 primer.

Fig. 2: PCR Amplification of TNF-α gene of Nili Ravi buffalo clinical mastitis using BT2 primer.

Fig. 3: PCR Amplification of TNF-α gene of Nili Ravi buffalo clinical mastitis using BT3 primer.

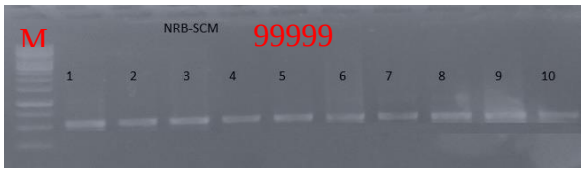

Fig. 4: PCR Amplification of TNF-α gene of Nili Ravi buffalo clinical mastitis using BT4 primer.

Fig. 5: PCR Amplification of TNF-α gene of Nili Ravi buffalo clinical mastitis using BT5 primer.

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

Sr. No	Position	Reference	SNPs	Results
1.	279	-	G	Insertion
2.	558	R	G	Silent
3.	1022	G	A	Transition
4.	1023	A	G	Heterozygous
5.	1129	G	A	Transition
6.	1059	Y	C	Silent
7.	1311	R	G	Silent
8.	1741	C	-	Deletion
9.	2091	G	A	Transition
10.	2065	Y	T	Silent
11.	2066	R	G	Silent
12.	2183	R	A	Silent

Fig. 6: PCR Amplification of TNF-α gene of Nili Ravi buffalo subclinical mastitis using BT1 primer.

Fig. 7: PCR Amplification of TNF-α gene of Nili Ravi buffalo subclinical mastitis using BT2 primer

Fig. 8: PCR Amplification of TNF-α gene of Nili Ravi buffalo subclinical mastitis using BT3 primer

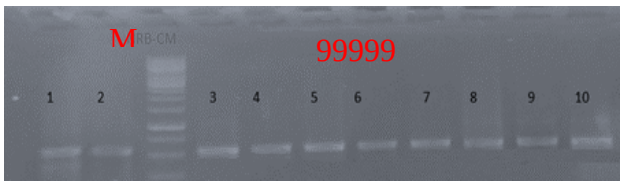

Fig. 9: PCR Amplification of TNF-α gene of Nili Ravi buffalo subclinical mastitis using BT4 primer

Fig. 10: PCR Amplification of TNF-α gene of Nili Ravi buffalo subclinical mastitis using BT5 primer.

Table 3: SNPs identified in TNF-α gene sequence of subclinical mastitis sample

Sr. No	Position	Reference	SNPs	Results
1.	279	-	G	Insertion
2.	558	R	G	Silent
3.	1022	G	A	Transition
4.	1023	A	G	Heterozygous
5.	1059	Y	C	Silent
6.	1311	R	G	Silent
7.	1741	C	-	Deletion
8.	2091	G	A	Transition
9	1129	A	G	Transition
10	2065	Y	T	Silent
11.	2066	R	G	Silent
12.	2183	R	A	Silent

Comparative analysis of TNF-α gene sequence of subclinical and clinically mastitic Nili-Ravi Buffalo showed the same mutations with other already reported sequence using NCBI blast except the transition of (A/G) at 1129.

Silent SNP's were also found in both clinical and subclinical mastitic samples as at position 558 reference indicated the presence of R which refers to purine but in all samples there were G. At position 1059 Y/C SNP was found. Position 1311 & 2066 showed same SNP change of R/G as at position 558. While site 2065 and 2183 indicated Y/R and R/A SNP's respectively.

Almost all these mutations were found in Intronic regions while some silent polymorphisms were there in exonic region, so none of them would affect the protein formation directly. So, it can be suggested that the mutations found in TNF-α gene have no significant effect on the formation of respective protein, while studies in cattle showed that there was a significant change in protein structure due to mutation present in TNF-α gene in cattle. High transitional frequency in exon 4 of TNF-α gene of Holstein-Friesian has also been observed by Shirasuna (2011) and Wojdak-Maksymie (2013). Similarly, Firyal *et al.* (2018), also found SNP *in* the exon 4 of TNF-α gene in Sahiwal cattle (Firyal *et al.*, 2018). No documented study has been found on the association of TNF-α gene Polymorphism with mastitis in Nili Ravi Buffaloes. So, it is difficult to establish a discussion with other studies on buffaloes with respect to the TNF-α gene Polymorphism association with mastitis. The findings of the present study, suggested that all these SNPs play an important role in immune function of the host and have an association with the risk of mastitis (Carvajal *et al.*, 2013).

In the present study, the complete TNF-α gene of Pakistani Nili Ravi buffaloes with clinical and without mastitis signs were amplified (Fig. 1, 2, 3, 4 and 5) and sequenced. Comparative analysis of this full length gene sequences and the reference sequence revealed 12 genetic variations at different locations (Table 2).

Conclusions: Novel mutations were found which were associated with TNF-α gene in clinical and sub clinical mastitic Nili Ravi buffalo. This study will help us in screening of mastitis resistant and susceptible buffalos. The findings of the present study will be very useful for improving mastitis resistance in dairy buffaloes by marker-assisted selection.

Authors contribution: SF: Problem identification, sampling, execution of experiments, data analysis and manusripct writeup. ST: Sampling, execution of experiments, data analysis and manusripct writeup. ARA: Data analysis and manusripct writeup. MT: Exprementaion and data analysis. MW: Data analysis and manusripct writeup. MN: Experimentaion. HS: Blood sampling and manusripct writeup. SN: Data analysis. SS: Samping. MMA: Manusripct writeup.

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