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Genetic polymorphism in *HSPB6* gene and their association with heat tolerance in Sahiwal cattle

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ABSTRACT

Heat shock proteins (HSPs) are known to modulate cellular response during summer stress in dairy cattle. Among different classes of HSPs, heat shock protein 20 (HSPB6) is a member of the small HSP family protein, the role of which has not been fully characterized in the context of heat stress in cattle. This study identified single nucleotide polymorphisms (SNPs) in the *HSPB6* gene in Sahiwal cattle and their associations with heat tolerance traits (RR, RT and HTC). Three SNPs (SNP 1-3) were reported, which included two transitions, viz. SNP1-g.436G>A (Intron 1) and SNP2-g.2152A>G (3′-UTR) and one transversion, viz. SNP3-g.2417A>T (3′-UTR). The association analysis revealed that SNPs loci, viz. SNP1-g.436G>A and SNP2-g.2152A>G were significantly associated with heat tolerance traits. The GG genotype of SNP2-g.2152A>G was significantly associated with heat tolerance traits in Sahiwal cattle. The association analysis of four available haplotypes, viz. Hap1 (GGA), Hap2 (AAA), Hap3 (GAA), and Hap4 (AAT) of *HSPB6* gene with heat tolerance traits did not differ significantly with any haplotype in Sahiwal cattle. This study provides the first association analyses between the SNPs of *HSPB6* gene and heat tolerance traits in Sahiwal cattle, which could be used as effective SNP markers in genetic selection for heat tolerance in cattle breeding programs.

Keywords: Haplotype, Heat shock protein B6, Heat stress, Sahiwal cattle, SNP

Heat stress in dairy cattle is a major cause of a decrease in production (West 2003), reproduction (Hansen 2004), and the inability of the immune system to fight disease (Boonstra 2004). The impact of heat stress on total milk production was estimated and predicted to be about 3.2 million tonnes in 2020 and more than 15 million tonnes in 2050 in India (Upadhaya *et al*. 2009). Nevertheless, due to global warming and climate change, the total loss of milk production in monetary terms amounts to a whopping 26616.2 million (135.01 $) annually in India (Upadhyay *et al.* 2009). Hence, the strategies for reducing the impact of heat stress on cattle productivity include modification of the micro-environment, nutritional management (Arjomandfar *et al.* 2010), and suitable breeding programs (Hoffmann 2010). Genetic variations in heat tolerance traits at the physiological and cellular levels are well documented by several studies on *Bos indicus* and *Bos taurus* cattle breeds (Hansen 2004, Lacetera *et al.* 2006, Sajjanar *et al.* 2013, Bhanuprakash *et al.* 2016, Deb *et al.* 2016).

Heat shock proteins (HSPs) are the transcriptional activation and accumulation of a set of proteins, which are categorized into different isoforms for their molecular weight, i.e. HSP27, HSP60, HSP70, HSP90, and HSP110/104 (Kregel 2002). These HSPs protect cells from heat and other stresses on their chaperone activity, which consists of supporting the non-covalent assembly and or disassembly of other macromolecular structures (Ellis 2006). Among all heat shock proteins, the *HSPB6*/*HSP20* gene belongs to the small molecular weight HSP family, first identified in 1994 as a complex with HSPB1 (Kato *et al.* 1994) in rat and human skeletons. The *HSPB6* gene has been mapped on *Bos taurus* autosome 18 (BTA-18) and spans nearly 2561 bp with 3 exons and 2 introns. HSPB6 is highly and constitutively expressed in different types of muscle including uterine smooth muscle (Cross and Dea 2007), cardiac muscle (Pipkin and Johnson 2003), and skeletal muscle (Tessier and Komalavilas 2004). The HSPB6 is considered to play an essential role, such as in intracellular protein transport and protecting the cytoskeletal architecture (Mymrikov *et al*. 2011). Moreover, increase in the phosphorylation of HSPB6 have been associated with cyclic nucleotide-dependent

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vasorelaxation in bovine carotid, bovine airway, porcine carotid, and porcine coronary smooth muscle tissues (Tessier and Komalavilas 2004, Komalavilas and Penn 2008). Therefore, this study was undertaken to investigate the polymorphisms in the *HSPB6* gene and their associations with heat tolerance traits in the Sahiwal cattle. The SNPs found in this study may be useful for further genetic improvement in this breed for thermotolerance.

MATERIALS AND METHODS

Experimental animals and geographical location: Sahiwal cattle (100) which were clinically healthy and completed at least one lactation were randomly selected at Livestock Research Complex (LRC), ICAR-National Dairy Research Institute, Karnal, Haryana (India), which is located at 29.68°N latitude and 76.98°E longitude with 235-252 m above mean sea level. The animals were kept in a herd under natural conditions according to animal welfare rules. They were fed on concentrate mixture, seasonal grass, paddy straw, and clean drinking water *ad lib*.

Data collection: The heat tolerance traits i.e. respiration rate (RR) and rectal temperature (RT) were measured continuously for four days during the intense hours of winter, spring, and summer seasons, and mean value was taken as final reading for association analysis. The heat tolerance coefficient (HTC) was estimated in accordance with heat tolerance index developed by Benezra (1954). Temperature humidity index (THI) was estimated for all days in three seasons, viz. winter, spring, and summer during which physiological parameters were recorded and taken as fixed variables in association analysis. THI was calculated using dry bulb temperature (D_b) and wet bulb temperature (W_b) to estimate the magnitude of heat stress (Thom 1959).

$$THI = 0.72(W_b+D_b) + 40.6$$

Genomic DNA extraction: In this study, 100 Sahiwal cattle were used for collection of blood samples (5-8 ml) from healthy and unrelated cattle using lithium coated vacutainer tubes containing heparin as an anticoagulant by jugular vein puncture under sterile conditions and were promptly transported to the laboratory under refrigeration. Genomic DNA was extracted from the blood leukocytes using phenol-chloroform extraction method (Sambrook and Russell 2001) and diluted to working concentration (30 ng/µL) and stored at -20°C, which were used as templates for polymerase chain reaction (PCR) amplification to explore SNP in *HSPB6* gene.

Primers and PCR amplification: The exons (1 and 3), intron 1, 3′-UTR, and flanking sequence of the cattle *HSPB6* gene were amplified from the genomic DNA samples. Three pairs of oligonucleotide primers were designed to amplify the cattle *HSPB6* gene using Primer 3 software (version 0.4.0) based on the bovine *HSPB6* gene sequence (GenBank Accession No. AC_000175.1). The primer sequence, product size, amplified region, and annealing temperature are listed in Table 1. PCR reactions were carried out in 25 µL volume containing 30 ng/µl genomic DNA, 0.5 µM of each primer, 12.5 µL PCR Master Mix (2×) (Fermentas) and 8.5 µL water. The PCR protocol was as follows: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 58.4-63°C for 45 sec, extension at 72°C for 1 min, and a final extension at 72°C for 5 min in a Bio-Rad T100 Thermal cycler. Subsequently, each PCR product (Fig. 1)

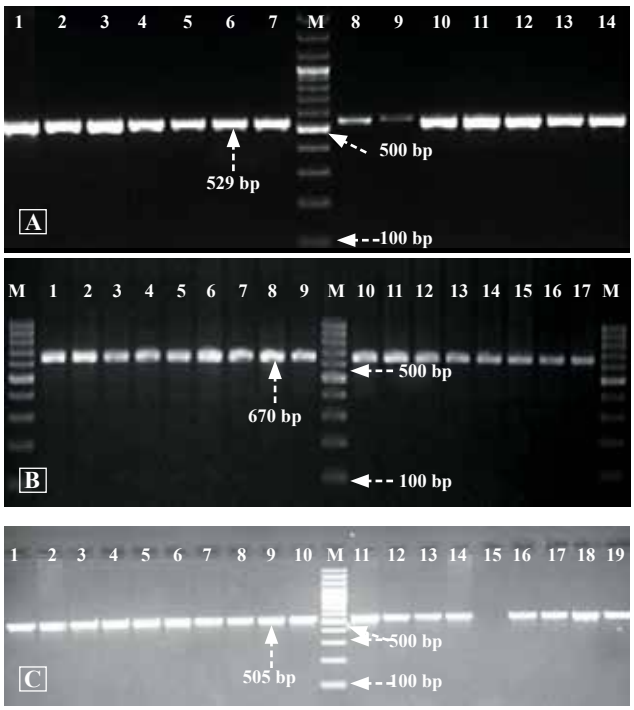

Fig. 1. PCR amplification products of *HSPB6* gene in Sahiwal cattle. **A.** Exon 1, Intron 1 and flanking sequence (529 bp); **B.** Exon 3 and part of 3′- UTR (670 bp); **C.** 3′-UTR and Flanking sequence (505 bp).

Table 1. PCR primer sequences of the *HSPB6* gene in Sahiwal cattle

Primer sets	Sequence (5′-3′)	Region amplified	Amplicon size (bp)	Annealing temperature (°C)
1.	F-GCGCTTAATAAATGGCCAAGTC R-GAGAATCCATACTCCGGCAAC	Exon 1, Intron1 and Flanking sequence (4..532)	529 bp	63.0°C
2.	F-GAGCCTCTCAAGCCTCTCTC R-TCCAGGGTGAGAGGTCTGT	Exon 3 and Part of 3′-UTR (1598..2267)	670 bp	61.0°C
3.	F-CCCTTCTCAAATGTCCAAGA R-CCTGGATCAGACATAAAGCA	3′-UTR and Flanking sequence (2223..2724)	502 bp	58.4°C

Note: F, Forward primer; R, Reverse Primer; bp, base pairs.

was detected by electrophoresis using a 1.5% agarose gel stained with ethidium bromide.

Statistical analysis: The population genetic indices viz. allelic frequencies, genotypic frequencies, effective allele number (ne), Shannon Index (I), expected heterozygosity (Nei), and polymorphism information content (PIC) were estimated by PopGene version 1.32 (Yeh *et al.* 1999). These SNPs of the HSPB6 gene were subjected to linkage disequilibrium (LD) analysis and haplotype analysis carried out by using Haploview 4.2 software (Barrett *et al.* 2005) and SHEsis platform (http://analysis.bio-x.cn/myAnalysis.php) (Shi and He 2005, Li *et al.* 2009). The associations of the SNPs with heat tolerance traits (RR/RT/HTC) were analyzed using the general linear model (GLM) procedure of SAS Version 9.2. The mixed statistical of the linear model analysis did not include the effects of herd, parity, and age of dam, which had no significant effects on the variation of traits.

The statistical linear model was:

$$Y_{ijk} = \mu + T_i + G_j + e_{ijk}$$

where Y_{ijk}, observation of the heat tolerance traits (RR/RT/HTC); μ, overall mean of each trait; T_i, effect of i^{th} THI (i = 48.77, 64.86, and 90.96); G_j, fixed effect of genotype or combined genotype/haplotype; and e_{ijk}, random residual error. Thus the fixed effect of genotype/haplotype and THI was a major source of variation. The mean±standard error (SE) was represented for all data and $P<0.05$ was considered a significant difference.

RESULTS AND DISCUSSION

Genetic polymorphism of HSPB6 gene in Sahiwal cattle: For determining SNPs in targeted region (exon 1 and 3, Intron 1, 3′-UTR, and flanking sequence) in *HSPB6* gene, reference sequence (NCBI GenBank: AC_000175.1) of *Bos taurus* available at NCBI genome browser were aligned with sequencing results of animals for each targeted region using ClustalW multiple alignment programs. Sequencing data were analyzed using Bio Edit software (version 7.2). After DNA sequencing and alignment analysis, three SNPs were detected, namely, SNP 1-3 (Fig. 2). The SNP1-g.436G>A locus was located at Intron 1 and mutated from G to A, resulting in transitional mutations, which were genotyped by the chromatogram analysis (Fig. 2a). The SNP2-g.2152A>G locus was located at 3′-UTR and mutated from A to G, which was genotyped by the chromatogram analysis (Fig. 2b). The SNP3-g.2417A>T locus was located at 3′-UTR and mutated from A to T, resulting in transversional mutations, which were genotyped by the chromatogram analysis (Fig. 2c).

Genetic parameter of HSPB6 gene polymorphisms: Frequencies of genotypes and alleles were different at different SNPs in Sahiwal cattle (Table 2). Only two genotypes of SNP1 and SNP3 loci, but three genotypes were found for SNP2 locus in Sahiwal cattle. Consequently, the population indexes of these SNPs loci were evaluated based on genotypic frequency numbers, including Ho, He, Ne, and PIC (Table 2). The classification of PIC values demonstrated that all SNPs viz. SNP1-g.436G>A, SNP2-g.2152A>G, and SNP3-g.2417A>T indicated a value of 0.420, 0.498 and 0.394, respectively, which collaborated with intermediate genetic diversity. The Sahiwal cattle population was observed to be away from Hardy Weinberg equilibrium and heterozygote deficiency was also observed.

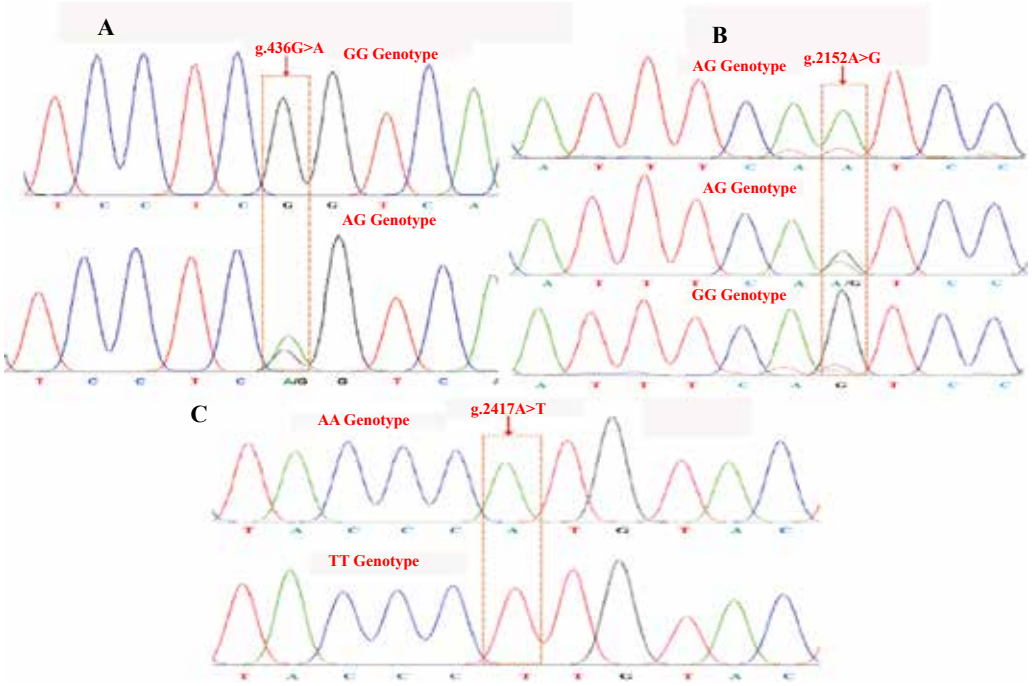

Fig. 2. Sequence chromas of three SNPs loci of *HSPB6* gene in Sahiwal cattle. **A.** Chromatograph of AC_000175.1: g.436G>A (SNP1); **B.** g.2152A>G (SNP2); **C.** g.2417A>T (SNP3). SNPs, single nucleotide polymorphisms; HSPB6, heat shock protein 6.

Table 2. Genetic parameters of the *HSPB6* gene in Sahiwal cattle

SNPs	Genotypes	Genotypic frequency	Allele	Allelic frequency	ne*	I*	Nei*	PIC*	HWE* test
SNP1-g.436G>A	AG	0.60	A	0.30	1.724	0.610	0.420	0.331	Disequilibrium
	GG	0.40	G	0.70					
SNP2-g.2152A>G	AA	0.39	A	0.53	1.990	0.690	0.497	0.374	
	AG	0.29	G	0.47					
	GG	0.32							
SNP3-g.2417A>T	AA	0.73	A	0.73	1.650	0.583	0.394	0.316	
	TT	0.27	T	0.27					

Note: ne*, Effective number of alleles; I*, Shannon's information index; Nei*, Expected heterozygosity; PIC*, Polymorphism information content; HWE, Hardy-Weinberg equilibrium.

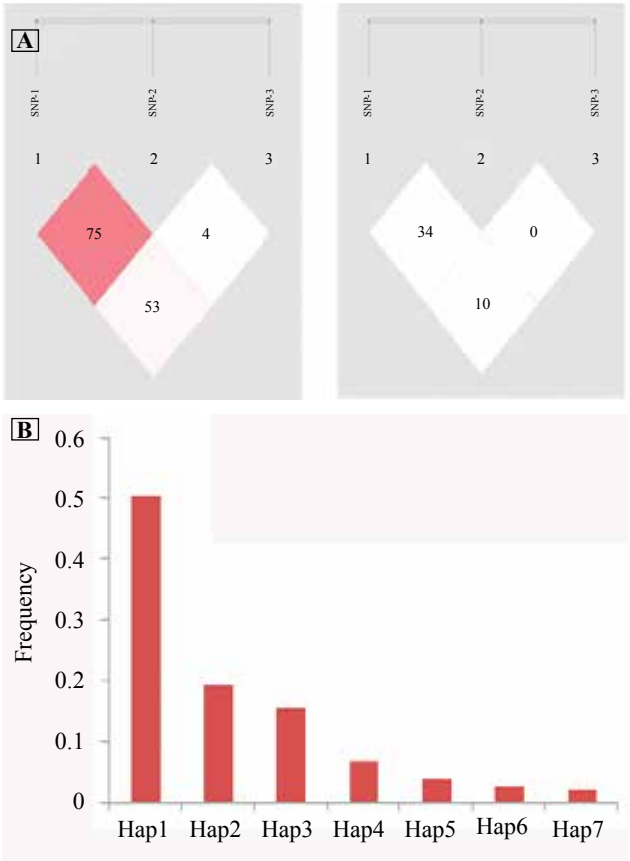

Fig. 3. Linkage disequilibrium (LD) and haplotypes frequencies of *HSPB6* gene in Sahiwal cattle. **A.** LD plot; **B.** Haplotypes frequencies.

Haplotype structure and linkage disequilibrium analysis: According to the D´ test and r^2 test in LD analysis, there was linkage disequilibrium among three SNPs (Fig. 3a and Table 3). The haplotype analysis was carried out by the Haploview and seven different haplotypes of *HSPB6* gene were found, which were Hap1 (GGA), Hap2 (AAA), Hap3 (GAA), Hap4 (AAT), Hap5 (GGT), Hap6 (AGT), and Hap7 (AGA) (Table 4). The frequency of the Hap1 (50.3%) was highest, and the Hap7 (2.0 %) was the lowest in Sahiwal cattle (Fig. 3b). The haplotypes with frequencies below 5% were meaningless in statistical analysis; hence haplotypes 5, 6, and 7 were not subjected to subsequent analysis (Table 4).

Table 3. D´ and r^2 values of pairwise linkage disequilibrium of the *HSPB6* gene in Sahiwal cattle

SNPs loci/D´	SNP1	SNP2	SNP3
SNP1	-	0.752	0.534
SNP2	-	-	0.047
SNP3	-	-	-
SNPs loci/ r^2			
SNP1	-	0.342	0.104
SNP2	-	-	0.000
SNP3	-	-	-

Table 4. Haplotype frequency within the *HSPB6* gene in Sahiwal cattle

ID of haplotypes	SNP1-SNP2-SNP3 g.436G>A- g.2152A>G-g.2417A>T	Haplotype frequency
Hap1	GGA	0.503
Hap2	AAA	0.190
Hap3	GAA	0.153
Hap4	AAT	0.066
Hap5	GGT	0.037
Hap6	AGT	0.025
Hap7	AGA	0.020

Note: HSPB6, Heat shock protein 6; SNP, single nucleotide polymorphism; Hap, haplotype.

Association between the genetic polymorphism and heat tolerance traits: The association analysis of the genetic polymorphism with heat tolerance traits is given in Table 5. The temperature humidity index (THI) was significantly ($P<0.01$) different for all heat tolerance traits, viz. RR, RT, and HTC.

HSPB6 SNPs and RR trait: Respiration rate (RR) has been used as an indicator of heat stress in cattle. When animals are exposed to the environmental temperature above the thermoneutral zone, the first reaction is the increase in respiration rate (Seath and Miller 1946). During heat stress, RR is an important thermoregulatory response, which aids in heat dissipation via evaporative cooling (Beatty *et al.* 2006). Results of a study by Srikandakumar and Johnson (2004) too indicated that due to an increase in respiration rate, there was greater metabolic heat production. Among three identified SNPs of *HSPB6* gene viz. SNP1-

Table 5. Least squares mean (LSMEANS) and standard errors (SE) for heat tolerance traits of different genotypes of HSPB6 gene in Sahiwal cattle

Effect	Sub-class	RR (times/min)	RT(°C)	HTC
Overall Mean		19.75±0.62	38.08±0.06	1.85±0.02
Seasons (THI)	Winter (THI=48.77)	14.09±0.43[a]	37.97±0.05[a]	1.60±0.01[a]
	Spring (THI=64.86)	18.56±0.43[b]	38.23±0.06[b]	1.80±0.01[b]
	Summer (THI=90.96)	26.61±0.41[c]	38.45±0.06[c]	2.16±0.03[c]
SNPs loci	Genotypes	RR	RT	HTC
SNP1-g.436G>A	GG (40)	19.70±0.58	37.34±0.07[a]	1.85±0.04
	AG (60)	19.78±0.45	38.13±0.10[b]	1.85±0.03
SNP2-g.2152A>G	AA (39)	19.52±0.60[b]	38.33±0.12[a]	1.84±0.04[b]
	AG (29)	21.13±0.62[a]	38.28±0.07[a]	1.91±0.02[a]
	GG (32)	18.78±0.68[b]	38.01±0.08[b]	1.80±0.03[b]
SNP3-g.2417A>T	AA (73)	20.05±0.73	38.30±0.09	1.87±0.51
	TT (27)	18.93±0.70	37.99±0.04	1.81±0.40

g.436G>A, SNP2-g.2152A>G, and SNP3-g.2417A>T, only one SNP viz. SNP2-g.2152A>G was significantly associated (P<0.01) with RR trait. The different genotypes of SNP2-g.2152A>G locus were categorized into three genotypes i.e. AA, AG, and GG, demonstrating that the genotype GG was significantly lower (P<0.01) than AG and AA genotypes with RR trait in Sahiwal cattle (Table 5).

HSPB6 SNPs and RT trait: Rectal temperature (RT) is an indicator of thermal balance and may be used to assess the adversity of the thermal environment which can affect the growth, lactation, and reproduction of dairy cattle (West 2003). In SNPs loci viz. SNP1-g.436G>A and SNP2-g.2152A>G, the different genotypes were found to be significantly (P<0.01) associated with RT (°C), which demonstrated that the genotype GG of SNP1-g.436G>A was superior to AG in Sahiwal cattle (Table 5). For the locus SNP2-g.2152A>G, the genotype GG was superior to AG and AA genotypes, demonstrating that the genotype GG was favoured for the RT trait in Sahiwal cattle.

HSPB6 SNPs and HTC trait: The associations of the genetic variations in the case of the derived parameter viz. HTC was found significantly associated (P<0.01) with SNP2-g.2152A>G. For the locus SNP2-g.2152A>G, the genotype GG was superior to AA and AG genotypes (Table 5), this shows that the genotype GG of Sahiwal cattle was too tolerable for heat stress. The effect of the remaining SNPs loci viz. SNP1-g.436G>A and SNP3-g.2417A>T were non-significant on the HTC trait (Table 5).

Association between haplotypes of HSPB6 and heat tolerance traits: Based on the haplotype analysis results (Table 4) and the frequencies of the combined genotypes or haplotypes, four available haplotypes, Hap1 (GGA), Hap2 (AAA), Hap3 (GAA), and Hap4 (AAT) were used to further evaluate the association with the heat tolerance traits of Sahiwal cattle. The association of four haplotypes with RR, RT, and HTC traits were not significantly different.

Heat stress produces an adverse effect on the reproduction and production of all domestic livestock, mainly dairy animals of high genetic potential (Sailo *et al*. 2015). When cells are exposed to heat stress, the expression of HSP families is induced (Morimoto and Santoro 1998). According to the critical role of HSPB6 in heat response, some studies focus on the properties of the multifunctional protective agent (Dreiza *et al.* 2010, Mymrikov *et al.* 2011). However, there were only a few comparative studies available on molecular characterization and SNP identification in *HSPB6* gene in Sahiwal and Karan Fries (*Bos taurus* × *Bos indicus*) cattle (Kumar *et al.* 2015, Kumar *et al.* 2017, Kumar *et al.* 2021). We screened polymorphism in targeted region of the HSPB6 and confirm whether the heat tolerance trait is associated with the polymorphism in the *HSPB6* gene in Sahiwal cattle.

In the present work, a total of three SNPs loci were identified in HSPB6 gene; two in the 3′- UTR (SNP2-g.2152A>G, SNP3-g.2417A>T), and one in Intron 1 (SNP1-g.436G>A) regions. Data analysis revealed that all genotype distributions in all three loci were in Hardy-Weinberg disequilibrium. Ho, He, Ne, and PIC were the indicator for evaluating the genetic parameter in population, the superior values of PIC and He, the higher levels of the genetic variation. It was intermediate polymorphism (0.25<PIC<0.5) at all SNPs loci in Sahiwal cattle. It could afford much genetic information and be suitable for linkage analysis between SNP markers and heat tolerance traits. Three SNPs (SNP1-g.436G>A, SNP2-g.2152A>G, SNP3-g.2417A>T) of HSPB6 were used for haplotype construction. The association of haplotypes with RR, RT, and HTC traits was not found significantly different.

Genetic variation may be used to heat tolerance breeding in Sahiwal cattle by marker-assisted selection (MAS). Verma *et al.* (2015) found cattle with GA genotype at g.507G>A locus of HSPB8 gene showed the desirable characteristics of low RT, which suggested that g.507G>A is a genetic marker of heat resistance traits in Sahiwal cattle. Kumar *et al.* (2015) reported homozygote animals with AA genotype at A1209G locus of HSP90AA1 had lower heat tolerance coefficient (HTC) (1.78±0.04[a]), as compared to AG and GG genotypes (1.85±0.03[b] and 1.91±0.02[c]) in Sahiwal cows. Moreover, Sailo *et al.* (2016) observed that

Sahiwal cows with the CT genotype at locus C1787061T of Hsp90ab1 gene exhibited a significant association (P<0.01) with RT trait. In other studies, it was found that allele T at SNP locus T4338C was found to be associated with lower RT in Thai indigenous, Frieswal and Sahiwal cattle (Charoensook *et al.* 2012, Sajjanar *et al.* 2015). Wang *et al*. (2013) observed GG genotype at g.651C>G locus of the *HSBP1* gene had desirable genotypes for genetic adaptability under thermal stress in Chinese Holstein cattle. Li *et al.* (2011) detected cows with TT genotype had lower PCE (P>0.01) at the 4,693 positions of HSF1 gene. Rong *et al.* (2019) reported that the AA genotype at g.616087A>G locus of HSF1 gene showed the desirable characteristics of low temperature (T), relative humidity (RH), and THI, which suggested that g.616087A>G is a genetic marker of heat-resistance traits in Chinese indigenous cattle.

In conclusion, three SNPs mutations were found, and one of them significantly affected heat tolerance traits, which could provide suggestions for molecular markers of heat tolerance that may be used for marker assisted breeding of Sahiwal cattle for better adaptation in the sub-tropical and tropical hot climate after validating in large number of samples.

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