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NOVEL SNPs DETECTION IN *HSPB6* GENE AND THEIR ASSOCIATION WITH HEAT TOLERANCE TRAITS IN BUFFALOES

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ABSTRACT

Heat shock proteins (HSPs) play a protective role during heat stress in dairy animals by acting as chaperones that facilitate the folding, unfolding, and refolding of denatured proteins. The present study was aimed to characterize *HSPB6* gene on molecular basis and to assess the association between detected single nucleotide polymorphism and physiological parameters in buffaloes. Ninety-six (96) female buffaloes reared at Directorate Livestock Farms (DLF), GADVASU were targeted in this study. *HSPB6* gene located on 18th autosome of *Bubalis bubalis* (BTA 18) plays an important role in apoptosis and stress response. Primers were designed as forward and reverse gene-specific primers with sizes of 578 and 720 bp, respectively. Two polymorphic loci (SNPs) which included translational mutation at locus A1277G in exon 2, and transversional mutation at locus A1123C in intron were found in *HSPB6* gene when contrasted to buffaloes (NCBI GenBank AC 059174.1). Nucleotide substitution at g.1277A>G in exon 2 was found to be non-synonymous which changed sequence at position 91 from lysine (K) to glutamic acid (E) and individual SNP locus i.e., g.1123A>C was found to be remarkably associated ($p < 0.01$) with respiration rate. It can be concluded that AG genotype at locus g.1277A>G showed minimum value of respiration rate, rectal temperature and heat tolerance coefficient which established them to be thermotolerant. Experimental study in large population is required to assess the relationship of allelic variations in key heat shock protein genes.

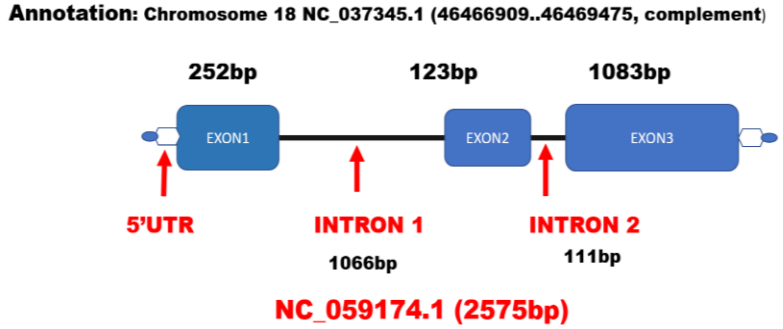
Keywords: Heat shock protein, *HSPB6*, marker-assisted selection, Murrah, Nili Ravi

INTRODUCTION

Resilience in animals is adversely affected by both short- and long-term environmental changes arising from factors such as temperature fluctuations, geographical conditions, dietary variations, and human-induced disturbances. Among these, heat stress and nutritional deficiencies are the most critical challenges affecting animal welfare, health, production, and reproduction, particularly in tropical regions (Niyas *et al.*, 2017). As a result, the productive potential of livestock is compromised, leading to reduced sustainability and increased economic burden. Domestic livestock, especially high-yielding dairy animals with superior genetic merit, are highly vulnerable to heat stress, which negatively impacts their reproductive efficiency, physiological stability, and production performance (Das *et al.*, 2016). Globally, there are 123 breeds of water buffalo, but only three breeds are widely distributed worldwide *viz.*, Murrah, Nili-Ravi, and Mediterranean Italian, due to their superior milk-producing ability (Minervino *et al.*, 2020). Due to their low heat tolerance and sparse sweat glands, buffaloes are more susceptible to heat stress. Heat shock protein (HSP) is one of the most significant

biomolecules involved in thermoregulation under stress (Ravaschiere *et al.*, 2017). This diversified family of HSP found in all kingdoms of life is highly conserved and divided into five subfamilies based on their molecular weight. These include small heat shock proteins (sHSPs), such as *HSPB6*, *HSP100*, *HSP90*, *HSP70*, and *HSP60*.

Genetic and genomic selection for enhanced heat resistance or physical stability may enhance resilience and animal welfare (Rexroad *et al.*, 2019). A polygenic multifactorial trait, heat resistance, is impacted by both genetic and epigenetic influences. The *HSPB6* gene family is a member of small heat shock protein (sHSP) family, which is a significantly conserved class of heat shock proteins produced by eukaryotes with chaperone activity. *HSPB6* is ubiquitous and simultaneously expressed in different types of muscles including uterine smooth muscle (Cross and Dea, 2007), cardiac muscle (Pipkin *et al.*, 2003) and skeletal muscle (Kato *et al.*,1994). It spans nearly 2575 bp and consists of three exons and two introns on *Bos taurus* autosome 18. The gene is generally localised in cytoplasm where it is present in association with *HSPB5* and *HSPB1* and *HSPB8*. The genomic organization of *HSPB6* gene is as illustrated below:



The association of SNPs in heat shock proteins and other candidate genes with physiological traits in livestock have been explored (Kumar *et al*., 2022a). For instance, polymorphisms in *HSP70*, *HSP90*, and *HSPB1* have been correlated with heat tolerance and reproductive efficiency in cattle and buffaloes (Hariyono *et al.*, 2022). However, there is scanty information on the role of *HSPB6* gene variations, warranting exhaustive explorations. Therefore, this study explored the natural responses of physiological traits during heat stress in Murrah and Nili Ravi buffaloes and ascertained their association with genetic polymorphisms in *HSPB6* gene. The present study was aimed to identify significant genetic markers that can be genetically leveraged to enhance thermotolerance and production in buffaloes.

MATERIALS AND METHODS

All the experimental procedures involving buffaloes were conducted in strict accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The study protocol was approval by the Institutional Animal Ethics Committee (IAEC) of Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana (GADVASU), Punjab, India (Registration No. 497/GO/Re/SL/02/CPCSEA dated 13 October 2021). Ninety-six (96) healthy adult females (of 46 to 76 months age in first to fourth lactating stage) of Murrah and Nili Ravi buffaloes maintained with *ad libitum* access to feed and water, and standard management at Directorate Livestock Farm, GADVASU, Ludhiana (Punjab) situated at30.9°N Latitude and 75.85°E longitude.

Measurement of recording physiological traits

Physiological traits such as respiration rate (RR), rectal temperature (RT, °C), and heat tolerance coefficient (HTC) of adaptability, were evaluated as indicators of heat stress response (Chikkagoudara *et al.*, 2022). Respiration rate and rectal temperature were recorded for all the animals in four seasons namely *viz*., winter, rainy, dry summer and wet summer three times consequently and averages taken as final reading for association analysis. These parameters were

recorded at the probable extreme hours of day i.e. 6-8 am and 12 noon-2 pm. The heat tolerance coefficient (HTC) was calculated using heat tolerance index developed by Benezra (1954) as per the following equation:

$$\text{HTC (Benezra coefficient of heat adaptability)} = \frac{RR}{23} + \frac{RT}{38.33}$$

wherein the denominator 23 and 38.33 were normal RR and RT under ideal conditions.
Lower the HTC value, higher is the degree of adaptability.

The temperature-humidity index (THI) was calculated for all days in four seasons *viz*., winter (50.96), rainy (76.51), hot humid summer (83.88) and summer (80.67) during which physiological parameters were recorded and used in the association analysis. The THI values were determined based on wet bulb (Wb) and dry bulb (Db) temperatures that was calculated as per National Research Council (NRC, 1971):

$$THI = 0.72 (Wb + Db) + 40.6$$

Primer designing and PCR amplification

The venous blood samples (5 mL each) were aseptically collected in EDTA tubes and stored at 4°C. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen). DNA quality was assessed by 1.5% agarose gel electrophoresis (Sambrook and Russell, 2001). Two sets of gene-specific oligonucleotide primers, each consisting of a forward and a reverse primer, were designed to amplify complete coding region of *HSPB6* gene (RefSeq: NC_059174.1) in buffalo. The primers were designed using Primer3 software (*https://primer3.org/*) and subsequently validated for specificity using the Basic Local Alignment Search Tool (BLAST) available through the National Center for Biotechnology Information (NCBI) (*https://blast.ncbi.nlm.nih.gov/Blast.cgi)***.** The selected primers were synthesized by Bioserve Genomics India Pvt. Ltd., Bangalore, India. The primer sequences, corresponding nucleotide positions, targeted genomic regions, and expected amplicon sizes for *HSPB6* were as under:

Primer sets	Target region	Amplicon size	Annealing temp.
Exon 1 (F) GCCTTGGAAGTAGTCGGGTTA=21 (R) GGGTGGATTAAAGGGACTGG=21	5'Region + Exon 1 and Intron 1	578 bp	63°C
Exon 2 and 3 (F)TAGGTTGGAAGGTTCAAGGCAC=22 (R) TCTGGGTAAAGAGGTCCTGGG=22	Intron 1, Exon 2, Intron 3 & coding region of Exon 3	720 bp	60°C

The PCR amplification was performed in a 25 µL reaction mixture comprising of 12.5 µL GoTaq Green PCR Master Mix (Promega), 8.5 µL nuclease-free water, 2.0 µL template DNA, and 1.0 µL each of forward and reverse primers. The thermal cycling protocol included an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 63 and 60°C for 45 sec each, and extension at 72°C for 60 sec (Bio Rad T100). This was followed by a final extension at 72°C for 5 min (1 cycle). Following amplification, PCR products were stored at 4°C until further analysis. Agarose gel electrophoresis was performed to verify the PCR amplification products, and the gels were visualized using a gel documentation system (BioRad). Each sequence was then aligned using ClustalW multiple sequence alignment software for comparative sequence analysis with *Bos taurus* and Ref Buffalo sequence (Accession No. ENSBBUG00015022258) taken from NCBI site (*https://www.ebi.ac.uk/Tools/msa/clustalw2/*).

Statistical analysis

The association of identified genetic variations with RR, RT and HTC in buffaloes was analysed using generalized linear model (GLM) of Statistical Analysis System (SAS) software (version 9.3) to identify any significant differences in a population.

$$Y_{ijklmno} = \mu + T_i + P_j + B_k + A_l + SNP1_m + SNP2_n + e_{hijklo}$$

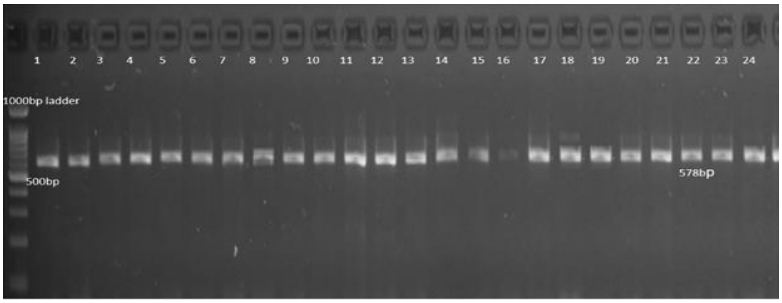
where, $Y_{ijklmno}$ = o^{th} observation on RR/RT/HTC of buffaloes with i^{th} THI, j^{th} parity, k^{th} breed, m^{th}

and n^{th} genotype, μ = Overall mean, T_i = Fixed effect of i^{th} THI (i = 1to 4), P_j = Fixed effect of j^{th} parity of animal (j = 1-7), B_k = Fixed effect of k^{th} breed (k = 1-2), A_l = Random effect of l^{th} animal (l = 1-96), $SNP1_m$ = Effect of m^{th} genotype of SNP1 A1123C (k = 1-3), $SNP2_n$ = Effect of n^{th} genotype of SNP 2 A1277G (k = 1-2) and $e_{ijklmno}$ = Random error associated with Y_{ijkl} observation and assumed to be NID (0, $\sigma^2 e$).

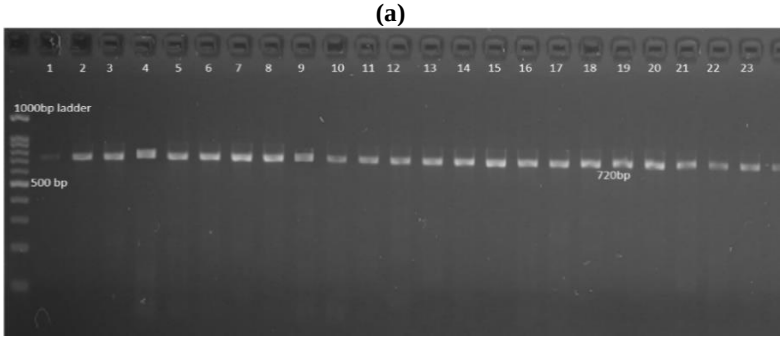
RESULTS AND DISCUSSION

The overall mean respiration rate (RR), rectal temperature (RT), and heat tolerance coefficient (HTC) were 21.22 ± 0.44 breaths min^{-1}, 37.99 ± 0.06°C, and 1.90 ± 0.02, respectively. Season had a highly significant effect ($p < 0.01$) on all three physiological traits. RR was highest during wet summer (24.55 ± 0.43) and lowest in winter (16.23 ± 0.44). A similar seasonal pattern was observed for RT, with significantly higher values during dry summer (38.28 ± 0.06°C) and the lowest RT recorded in winter (37.51 ± 0.06°C). HTC values were also significantly elevated during wet summer (2.06 ± 0.01) indicating greater heat stress, whereas the lowest HTC was observed in winter (1.68 ± 0.01). In contrast, parity had no significant effect ($p > 0.05$) on RR, RT, or HTC, with values remaining relatively uniform across parities.

Agarose gel electrophoresis of amplicons revealed an amplification of a fragment size of 578 and 720 bp in *HSBP6* gene (Fig. 1). Further sequencing revealed exon 1 to be monomorphic, revealing no change in nucleotide arrangement in comparison to the reference sequence, however exon 2 had two SNPs, one transversion at g.1123 A>C (SNP 1) in the intronic region and the other transition at g.1277A>G (SNP 2) in the coding area (Fig. 2). However, comparative sequence analysis in Karan Fries (*Bos taurus x B. indicus)* cattle reportedly have 5 nucleotide polymorphisms *viz*., g.161A>G (flanking region), g.436G>A (intron 1) and g.2152A>G (3' untranslated region) loci (transitional mutations) and g.1743C>G (3′UTR) and g.2417A>T (3′ UTR) loci (transversional mutations) in *HSPB6* gene as compared to *B. taurus* (NCBI GenBank AC_000175.1) (Kumar *et al.*, 2017). Kumar *et al.* (2015) identified two transitions, g.436G>A and g.2152A0>G, and one transversion, g.2417A>T, within 3' untranslated region (UTR) and intronic region of *HSPB6* gene in Sahiwal cattle. Also, the exon 2 exhibited a mono-morphic pattern. These differences may be attributed to species- or breed-specific genetic variations, differing selective pressures, or population-specific diversity. Notably, exon 2 is mono-morphic in Sahiwal cattle (Kumar *et al.*, 2015). The present study identified a non-synonymous SNP (g.1277 A>G) in Murrah buffalo.



(a)



(b)

Fig. 1: Agarose gel electrophoresis of amplicons: Lane 1 has 1000 bp DNA ladder, while lanes 2–23 show PCR products from buffalo samples with a single specific band of 578 bp (a) and (b) 720 bp.

Association between genetic polymorphism and heat tolerance traits

The association analysis of genetic polymorphism with heat tolerance traits is given in Table 1. The

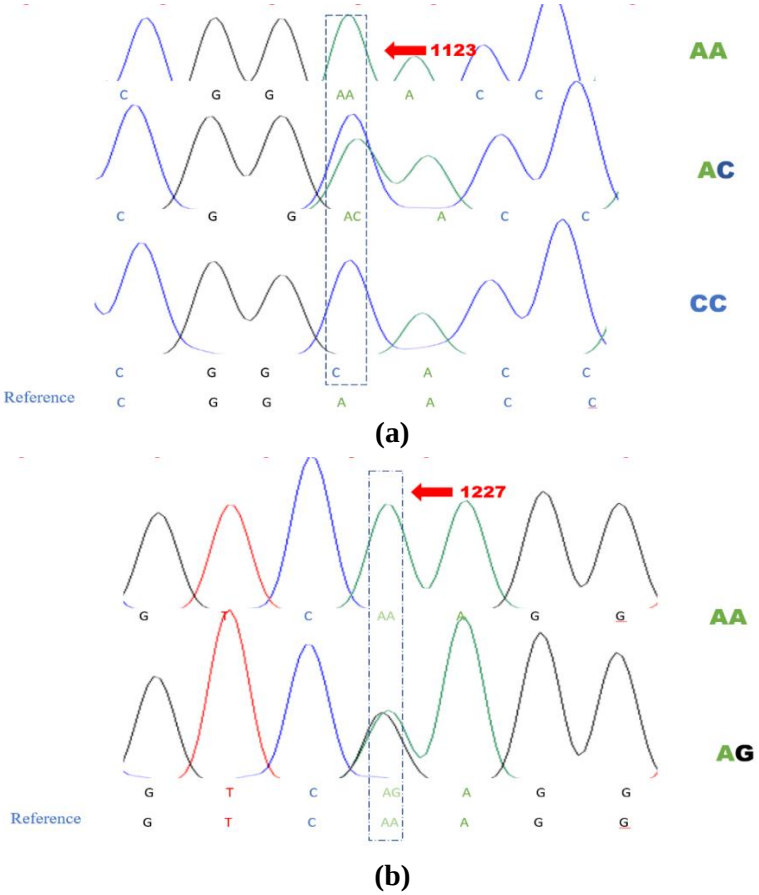


Fig. 2: Chromatogram showing SNPs at position a) g.1123A>C of *HSPB6* gene b) g.1277A>G of *HSPB6* gene in buffalo

THI, which reflects the impacts of both temperature and relative humidity, is frequently used to estimate heat stress experienced by dairy buffaloes. When the ambient THI exceeds 72, there was a hazard of heat stress in buffaloes. The temperature humidity index (THI) differed significantly ($p < 0.01$) for all heat tolerance traits, *viz*., RR, RT, and HTC. Statistical analysis using a mixed model in the animals having fixed effect such as season, breed, parity as well as random effect (animal) indicated that highly significant effect of breed ($p < 0.05$) and season ($p < 0.01$) on dependent variable i.e., HTC and RR while only season was found significant ($p < 0.01$) in case of RT in both Murrah and Nili Ravi animal population included in the present study. Least square means (Ls means) for Nili Ravi on HTC (1.929 ± 0.018), RT (38.02 ± 0.06°C) and RR (21.553 ± 0.437 breaths min^{-1}) was high as compared to Murrah buffalo population having HTC, RT and RR value of 1.899 ± 0.019, 37.97 ± 0.06°C and 20.900 ± 0.450 breaths min^{-1}, respectively (Table 1). Increased RT and RR physiology directly resulted from sun exposure as they were reared in an extensive pasture system. Genotypic analysis revealed that at the A1123C locus, rectal temperature differed significantly among genotypes ($p < 0.01$), with AA genotype exhibiting the highest RT and the CC genotype the lowest. In contrast, genotypic differences for RR and HTC at this locus were insignificant ($p > 0.05$), suggesting comparable physiological responses among genotypes for these traits. At A1277G locus, no significant associations ($p > 0.05$) were observed between genotypes and RR, RT, or HTC. Previous studies on *HSP70* and *HSP90* genes have demonstrated significant associations with thermoregulatory traits such as rectal temperature, respiration rate, and heat tolerance indices (Shenhe *et al.*, 2018). In *HSP70*, polymorphisms have been widely reported across diverse buffalo populations, including Murrah buffaloes (Kumar *et al.*, 2020; Singh *et al.*, 2020), Nili-Ravi × Murrah crossbreds and Mediterranean buffaloes (Shenhe *et al.*, 2018), as well as water buffaloes (Maylem *et al.*, 2025). Comparable associations have also been documented in Bali cattle (Suhendro *et al.*, 2024) and in crossbred cattle and riverine buffaloes (Sharma *et al.*, 2023), highlighting the conserved role of *HSP70* in heat stress adaptation across the species. Similarly, *HSP90* has been studied in crossbred populations, including Nili-Ravi × Murrah crossbreds and Mediterranean buffaloes

Table 1: Least square means of subclasses of different fixed effects for respiration rate, rectal temperature and heat tolerance coefficient of *HSPB6* gene in buffaloes

Effects	Subclass	Respiration rate (No. of breaths min^{-1})	Rectal temperature (^oC)	Heat tolerance coefficient
Overall mean		21.22 ± 0.437	37.99 ± 0.06	1.90 ± 0.018
Seasons (THI)	Dry summer (THI = 80.67)	24.40 ± 0.44^b	38.28 ± 0.06^a	2.05 ± 0.01^b
	Rainy (THI = 76.51)	19.71 ± 0.44^c	38.00 ± 0.06^c	1.84 ± 0.01^c
	Winter (THI = 50.96)	16.23 ± 0.44^d	37.51 ± 0.06^d	1.68 ± 0.01^d
	Wet summer (THI = 83.88)	24.55 ± 0.43^a	38.18 ± 0.06^b	2.06 ± 0.01^a
Parity	1	21.14 ± 0.44	37.98 ± 0.06	1.91 ± 0.01
	2	21.28 ± 0.42	38.00 ± 0.06	1.91 ± 0.01
	3	21.17 ± 0.48	37.99 ± 0.07	1.91 ± 0.02
	> 3	21.29 ± 0.44	37.99 ± 0.06	1.91 ± 0.01
Breed	Murrah	20.90 ± 0.45	37.97 ± 0.06	1.89 ± 0.01^b
	Nili Ravi	21.55 ± 0.44	38.02 ± 0.06	1.92 ± 0.01^a
Polymorphic loci				
A1123C	CC	20.65 ± 1.185	37.66 ± 0.16^c	1.88 ± 0.05
	AC	21.61 ± 0.263	38.15 ± 0.03^b	1.93 ± 0.01
	AA	21.41 ± 0.179	38.17 ± 0.02^a	1.92 ± 0.00
A1277G	AG	21.14 ± 0.508	37.99 ± 0.07	1.91 ± 0.02
	AA	21.31 ± 0.399	38.00 ± 0.05	1.91 ± 0.01

The values superscripted with same letters in the same column do not differ significantly from each other.

(Shenhe *et al.,* 2018), further reinforcing its critical role in physiological responses to heat stress. Contrarily, Chen *et al.* (2025) observed insignificant increase in RR in *HSP70* and *HSP90* in summer than in winter season. Conversely, the rectal temperature was significantly higher in winter than in summer ($p < 0.05$). This may be attributed to the presence of shade canopies and flowing water in the experimental area, allowing the buffaloes to find cool spots to decrease their body temperature. In addition to this, polymorphism and its association with physiological traits have been reported in sHSPs, *HSPB8* (Verma *et al.,* 2016), *HSPB1* (Saikia *et al.,* 2020) and *HSPB6* (Kumar *et al.,* 2022a,b). These studies underscore the role of heat shock proteins in heat stress response in buffaloes.

Conclusions: Two mutations and two reported genotypes associated with each SNP were identified in buffaloes. Exon 1 was found to be monomorphic as compared to the reference sequence whereas a total of 2 SNP at g.1123 A>C in intronic region and g.1277A>G and in coding region of exon 2 were identified. CC and AG genotypes recorded least RR, RT and HTC are better thermotolerant as compared to remaining genotypes. Therefore, it is suggested that further research on *HSBP1* gene is necessary to conclude its role in thermoregulatory function in animals.

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Ethical statement: All the experimental procedures involving buffaloes were conducted in strict accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The study protocol received prior approval from the Institutional Animal Ethics Committee (IAEC) of Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India.

Conflict of interest: There is no conflict of interest involved in this work.

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